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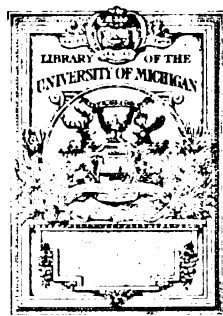
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U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY.—BULLETIN 111.

A. D. MELVIN, CHIEF OF BUREAU.

A CHEMICAL AND PHYSICAL STUDY OF
THE LARGE AND SMALL FAT
GLOBULES IN COWS' MILK.

BY

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AND

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1909.

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LETTER OF TRANSMITTAL.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY,
Washington, D. C., October 20, 1908.

SIR: I have the honor to transmit herewith, and to recommend for publication in the bulletin series of this Bureau, the accompanying manuscript of an article entitled "A Chemical and Physical Study of the Large and Small Fat Globules in Cows' Milk," by R. H. Shaw, of the Dairy Division of this Bureau, and C. H. Eckles, of the Missouri Agricultural Experiment Station. This work is a preliminary part of a comprehensive investigation being conducted at the Missouri station by cooperation between that station and the Dairy Division concerning the effects of period of lactation, feed, and other factors on the chemical composition of milk.

At the request of the authors acknowledgment is made to Messrs. J. O. Halverson and G. C. Payne for assistance in the chemical and microscopical work.

Respectfully,

A. D. MELVIN,
Chief of Bureau.

Hon. JAMES WILSON,
Secretary of Agriculture.

CONTENTS.

	Page.
Introduction.....	5
Previous investigations.....	5
Experimental work.....	8
Source of the samples	8
Method of separation	8
Preparation of fat samples for analysis.....	10
Analytical methods.....	10
Color determinations	11
Microscopical work	11
Results of experiments	14
Conclusion.....	16

ILLUSTRATIONS.

	Page.
FIG. 1. Showing method of counting globules.....	12
2. Showing the average relative size of large and small fat globules.....	13

A CHEMICAL AND PHYSICAL STUDY OF THE LARGE AND SMALL FAT GLOBULES IN COWS' MILK.

INTRODUCTION.

In planning a long investigation on the effect of the period of lactation, feed, and other factors on the chemical composition of milk it was decided that the most practical way of extracting the fat from the milk was by passing it through a small separator and churning the cream. In this method the tendency is for the smaller globules to escape in the skim milk and buttermilk; hence it became necessary to ascertain whether or not these small globules which in part escape differ in chemical composition from the large globules, and if such a difference exists, to determine just what it is.

Investigations on these points are not lacking, and had these investigations been sufficiently extensive, or had they agreed on the principal points, the work herein presented would have been unnecessary. However, such is not the case. There is almost a total lack of agreement in the work hitherto published, as will be seen later on. When the present investigation was taken up it was the intention to cover only those points necessary to validate certain determinations involved in the long investigation mentioned above. Later it was decided to enlarge it to some extent and publish it as a contribution to the knowledge of the chemistry of butterfat.

PREVIOUS INVESTIGATIONS.

Müller^a (1867) observed that fat extracted from skimmed milk is waxlike, and also that in the analysis of butter where 100 grams or more of fat-free substance is obtained the last particles of fat extracted have more the properties of wax than of butterfat.

Schroeder^b in 1872 published some investigations showing that when milk is fractionally creamed the butterfat obtained from the

^a Müller, Alexander. *Chemische untersuchungen auf dem gebiete der milch-wirtschaft. Die Landwirthschaftlichen Versuchs-Stationen.* Band 9, pp. 364-396. See p. 390. Chemnitz, 1867.

^b Schroeder, G. *Beiträge zur kenntniss des fettgehalts der milch.* *Milch Zeitung.* No. 28, pp. 325-327, 1872.

first fraction has a lower melting point and lower specific gravity than the butterfat obtained from the last fraction. He also stated that the latter contained no butyric acid.

Klusemann^a in 1893 conducted an investigation with milk from individual cows. He separated the large and small fat globules by passing the whole milk, at a temperature of 5 to 12° C., through a De Laval separator running at low speed. The cream contained the large globules. The skim milk was then warmed somewhat and again passed through the separator running more rapidly. The cream in this instance contained the medium-sized globules. The small globules were obtained by heating the last skim milk to 36 to 38° C. and again passing it through the separator, this time running very rapidly; the cream was churned in a small dash churn, and the resulting butter was melted and filtered by means of a jacketed filter.

He determined the melting and freezing points, the water-insoluble fatty acids, the melting and freezing points of the insoluble fatty acids, the iodine absorption number, the volatile acids, and the specific gravity. In his conclusions he states that the percentage of the insoluble fatty acids, and the melting point and freezing point of the butterfat, and of the insoluble fatty acids are higher in fat from small-globule cream than in fat from large-globule cream, the reverse being true of the percentage of volatile acids and of the iodine number.

Gutzeit^b (1895) published an investigation on the same subject. He chose mixed milk from a herd of 150 cows. He separated the large and small globules as follows: He used 100 liters of milk, 20 liters of which was allowed to rise for six hours in a pan immersed in cold water. The resulting cream contained the large fat globules. The remaining 80 liters was then run through a separator, the cream discarded, and the skim milk coagulated. The whey was then run through a separator, and the resulting whey cream contained the small globules. The two creams were churned separately, and the resulting butters were heated for a time at 60° C. and filtered. He determined the specific gravity, the melting point, the refractive index, insoluble fatty acids, volatile fatty acids, saponification number, and iodine absorption number, and concludes that in homogeneous milk the fat globules of all sizes have the same chemical-physical composition.

^a Klusemann, Erich. Die Zusammensetzung und die Beschaffenheit der aus den grossen und den kleinen fettkügelchen der kuhmilch gewonnenen butter. Inaugural dissertation. Leipzig, 1893.

^b Gutzeit, Ernst. Die schwankungen der mittleren grösse der fettkügelchen in der kuhmilch nach laktation, fütterung und rasse, sowie über den physikalischen und chemischen unterschied der grössten und kleinsten fettkügelchen. Landwirtschaftliche Jahrbücher, Bans 24, pp. 539-668. Berlin, 1895.

Lemus^a (1902) separated the large and small globules by interrupted milking. The first liter of milk drawn he took to represent the small globules and the last fraction of about one-half liter drawn the large globules. After reserving a sample for fat determination and microscopical examination, he extracted the fat according to the method of Soxhlet by shaking with ether after having first made it alkaline with potassium hydroxid. The ethereal layer was then removed and dried at 100° C. to a constant weight. He made some of the same determinations as did Klusemann and Gutzeit, but in his conclusions refers only to the volatile acids and the oleic acid. He states that in the majority of cases the smaller fat globules contained more oleic acid and less volatile acids than did the larger globules.

In the three more recent investigations, that is, those by Klusemann, Gutzeit, and Lemus, it is seen that no two draw the same conclusions. Gutzeit claims that there is no difference, chemically or physically, between the large and small fat globules in the same milk. Klusemann and Lemus agree in claiming that there is a difference, but disagree on some of the points of difference.

Klusemann worked on the milk of five individual cows and on the mixed milk of seven. A study of his results shows that in every case the percentage of insoluble acids in small-globule fat is higher than in the large-globule fat, the difference being from about 0.3 per cent to about 3 per cent; the melting point of the butterfat was higher in the small-globule fat than in the large, the variation being from 0.5 to 2° C.; the volatile fatty acids in terms of Reichert-Meissl number showed a decrease in the small-globule fat in six cases and an increase in two cases, the variation being from about 1 c. c. to about 4.5 c. c.; the iodine number in three cases showed practically no difference, in three cases it showed a decrease in the small-globule fat and in one an increase, the largest difference being about 2.5 per cent. Granting that no criticism can be made of his methods and work, it would seem that with the limited number of animals used his conclusions even on the insoluble fatty acids and melting point are unwarranted, and certainly not justified in the case of the other constants.

In Lemus's investigation it may be seen that the separation of the large and small globules is not positive. In fact, instead of the relative size of the fat globules being invariably larger in the stripper milk than in the foremilk, the reverse is true in some cases. In 10 cases the iodine number was smaller in the large-globule fat than in the small-globule fat, and in 3 cases it was larger, the difference ranging

^a Lemus, Woldemar. Ueber die chemische beschaffenheit des in den grossen und in den kleinen milchkügelchen enthaltenen fettes. Inaugural dissertation. Leipzig, 1902.

from 0.11 to 3.54 per cent. With the volatile acids the Reichert-Meissl number was smaller in 16 cases in the large-globule fat than in the small-globule fat, and in 6 cases the reverse obtained, the differences ranging from 0.45 to 13.85 per cent. It is also doubtful whether the fat remained unchanged in the preparation of the samples, where a prolonged heating at 100° C. was necessary to eliminate the ether.^a Moreover, it would seem that his determinations show the difference between the fat in the foremilk and that in the milk secreted during the process of milking, rather than that between the large and small fat globules in the same milk.

EXPERIMENTAL WORK.

For our investigation animals were selected which exhibited a wide range of breed, age, and period of lactation. In the earlier part of the investigation the chemical and microscopical work was limited to determinations of the relative size of the fat globules, the iodine number, the Reichert-Meissl number, the Koetstorfer number, and the refractive index. Later it was extended to include the specific gravity, the melting point, the Hehner number, the color, and the percentage of different-sized fat globules.

Table 1 gives the data concerning the animals. In Tables 2, 3, and 4 will be found the results of the chemical and microscopical work.

SOURCE OF THE SAMPLES.

The cows selected to supply the samples represented four breeds—Jersey, Holstein-Friesian, Ayrshire, and Shorthorn—and were members of the Missouri Agricultural College herd. Groups supplying the samples were arranged with a view of having certain samples represent milk from cows in the early part of the period of lactation, while others represented milk produced in the latter part of the period. The table shows the number of days in milk, the breed, the age, the date at which samples were taken, and the average percentage of fat in the milk of each cow. The sample of milk taken was the total product for one day of the group of cows shown in the table.

METHOD OF SEPARATION.

The first method tried consisted in allowing the milk to stand at room temperature for about two hours until a portion of the cream had come to the surface, when it was removed by drawing the remainder of the milk from a faucet in the bottom of the can. The partially

^a According to C. A. Brown (Annual Report of the Pennsylvania State College, 1899-1900, p. 208), a temperature much above 50° C. will very soon alter the composition of butterfat. Butterfat kept at 50° C. for two days showed a loss of over one unit in iodine number.

skimmed milk secured in this way was again placed in the can and allowed to stand about twelve hours, when the second portion of the cream was removed as before. This skim milk, which still contained approximately 0.3 per cent of fat, was then run through a centrifugal separator at a temperature of 32° C. The cream thus secured contained the smallest fat globules which it is possible to obtain by the centrifugal machine. The cream secured from the first skimming contained the larger fat globules, as they reach the surface first under the influence of gravity, but it also contained some small fat globules. In order to eliminate these this cream was placed in the bottom of a deep, narrow can, which was then filled with the skim milk from the final separation with the centrifugal separator. When most of the cream had again risen it was skimmed off and retained for the sample of the large fat globules.

The above method was found to be satisfactory, but equally good results were secured, at a considerable saving of time, by using the centrifugal separator. After several preliminary trials the following plan was adopted:

The milk was first run through the centrifugal separator at a temperature of 30° to 32° C. and the speed of the machine so regulated by trial that approximately one-half of the fat in the original milk was taken out as cream and one-half remained in the skim milk. It was found that with a hand-power separator, the normal speed of which was 60 revolutions of the crank per minute, this result was obtained when the machine was operated at the rate of 15 to 25 revolutions a minute, depending upon the character of the milk separated. The cream secured from this separation contained the larger fat globules, but also a large number of medium sized and some small ones, which were removed as described later.

The skim milk secured by this separation contained approximately one-half the fat originally found in the milk, and was again separated at a higher speed—from 29 to 40 revolutions of the crank to the minute. The speed was adjusted after trial with each sample of milk so that about 0.2 per cent of fat remained in the skim milk. The cream from this second separation was rejected, as it contained medium sized and some large fat globules which had escaped the first separation.

The skim milk containing approximately 0.2 per cent of fat was then heated to 38° C. and again run through the centrifugal separator at a speed of from 65 to 70 revolutions of the crank to the minute. The cream from this separation contained the smallest fat globules which it is possible to secure with a centrifugal separator. It is impossible to obtain all of the fat by any mechanical method of separation. The fat remaining in the skim milk after this final separation was found to be from 0.025 to 0.004 per cent, as shown by the Babcock method, using the double-necked Wagner testing bottle.

The cream from the first separation containing the largest fat globules was added to the skim milk from the third and run through the separator again at the speed used in the first separation, to eliminate the smaller fat globules which had remained with the large. The cream from this separation was again mixed with a second lot of the same skim milk from the third separation and the process repeated, this cream being taken as the final sample containing the largest fat globules. The two lots of cream were then churned by shaking in a closed jar as long as any butter could be accumulated.

PREPARATION OF FAT SAMPLES FOR ANALYSIS.

The butter was immediately melted on a steam bath and allowed to remain in this condition until the curd and water had settled. Special care was taken at this point that the temperature did not rise above 50° or 55° C. and also that it remained, even at this temperature, no longer than was necessary. It was then filtered through a paper filter kept warm by an electrical device, and preserved in corked bottles which were protected from light in a refrigerator until the butter was analyzed.

ANALYTICAL METHODS.

The methods of the Association of Official Agricultural Chemists were employed whenever possible. Duplicate determinations were made in all cases and in some triplicate or even quadruplicate. Only the briefest description of the official methods used is given below. For a detailed description reference may be made to Bulletin 107 of the Bureau of Chemistry, United States Department of Agriculture.

Specific gravity.—The specific gravity was determined in small pycnometer bottles at the temperature of boiling water.

Melting point.—The melting point was determined according to Wiley's method, by placing a disk of the fat in a large test tube containing boiled distilled water and boiled alcohol which had been cooled, the tube being placed in a beaker of water which was slowly heated, and noting, by means of a thermometer graduated to 0.1° C., the temperature at which the disk assumed the form of a sphere.

Refractive index.—The refractive index was determined with a Zeiss-Abbe refractometer which had been standardized with distilled water. The fat was kept at a constant temperature above its melting point during the determination by means of a current of warm water circulating through the instrument. The reading was reduced later by means of a factor to 25° C.

Volatile acids.—The method of Reichert, modified by Meissl, was employed in the estimation of the volatile acids, and the results are given as Reichert-Meissl numbers. In saponifying, use was made of the Leffman-Beam method, in which the fat is saponified in a flask

over a naked flame with a mixture of a strong aqueous solution of caustic soda and pure glycerol. The soap obtained in this way is decomposed by sulphuric acid and the liberated volatile acids are distilled over with the steam and titrated with decinormal barium hydroxid solution.

Saponification value.—The saponification value, or Koettstorfer number, was determined by the regular official method. The fat is saponified in a flask on a steam bath with an alcoholic potash solution, using a long glass tube as reflux condenser.

Iodin absorption number.—The method of Hübl was employed in this case. The fat is dissolved in chloroform and subjected to the action of a mixture of an alcoholic solution of iodine and of mercuric chlorid in a dark place for three hours, at the end of which time the unabsorbed iodine is determined by titrating with standard sodium thiosulphate solution. The percentage of iodine absorbed by the fat is expressed in the results as the iodine number.

Insoluble fatty acids.—For this determination the official method, or that of Hehner, as it is called, was employed. It was found, however, that better results were obtained by increasing the amount of fat to 10 grams, as suggested by Brown.^a The results are given as Hehner numbers.

COLOR DETERMINATIONS.

The color determinations were made with the Lovibond tintometer, using the standard color glasses. While this instrument is not admirably adapted to the work with milk and cream, so far as the authors' knowledge goes, it is the best that is available. With the melted fat where the light is transmitted through a considerable layer the determinations can be made quite accurately, but with the opaque milk, cream, and butter, where the light must be reflected from the surface, very slight differences in color can not be determined. The results are given in terms of the numbers assigned to the standard color glasses.

MICROSCOPICAL WORK.

In the microscopical part of the work the method devised by Dr. S. M. Babcock^b was used. The milk is diluted with distilled water to fifty times and the cream to one hundred times its volume. Fine capillary tubes are drawn out from larger tubes, care being taken that the resulting tubes are round in cross section. They are then broken into pieces 2 to 3 centimeters in length, and by means of forceps each tube is filled by immersing one end in the diluted cream or milk. The tube fills instantly by capillary attraction.

^a Report of Pennsylvania State College, 1899-1900, p. 211.

^b Fourth Annual Report of the New York Agricultural Experiment Station, 1885.

The two ends are then sealed with vaseline. Three such tubes are used for each sample, and these are laid parallel on a slide. A drop of glycerin is then placed over the tubes and a cover glass applied. The slide thus prepared is allowed to remain on a leveled slab for half an hour, the purpose of this being to allow the globules to rise to the top of the capillary tubes. At the end of thirty minutes the slide is placed under a microscope provided with an ocular micrometer and a mechanical stage. The number of globules in 50 divisions of the micrometer scale are counted in three places in each tube, the internal diameter of the tube at each place of counting being first deter-

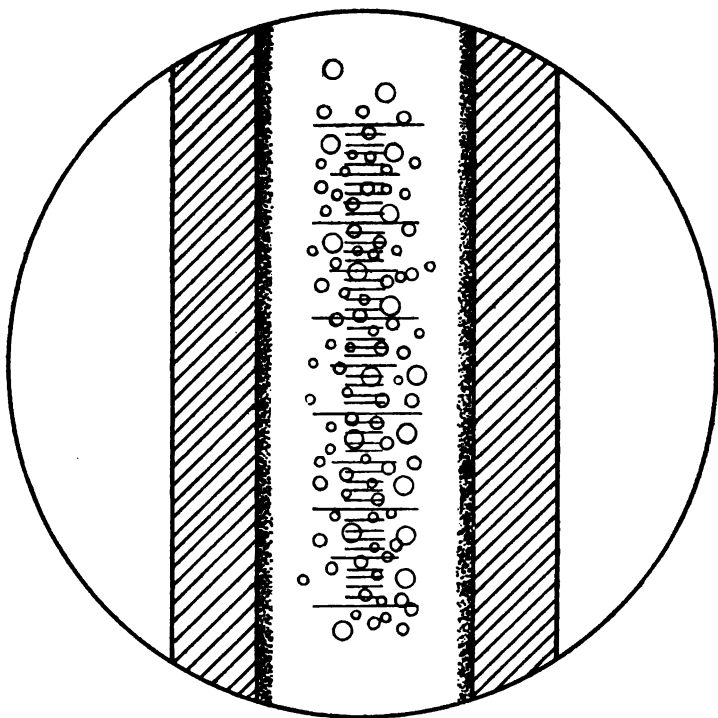


FIG. 1.—Showing method of counting globules.

mined by throwing the ocular micrometer scale across the tube at right angles and carefully counting the divisions within the two walls. The total number of globules in the 50 divisions is recorded, as well as the number under one division in diameter; also the number between one and two divisions in diameter, and the number having a diameter greater than two divisions. A 1-inch ocular and one-sixth inch objective are employed, and with this combination the value of each division of the ocular micrometer with the instrument used is 0.00258 millimeter. In this way nine determinations are made for each sample.

In order to compare results, the number of globules which would be found in a standard tube 100 divisions in diameter and 50 divisions in height is calculated from the number in each observed tube and the average taken. In making this calculation the formula $\frac{10,000 n}{d^2}$ is used.^a With the ocular micrometer used in this work the standard tube would have a volume of 0.006744 cubic millimeter, and if the milk is diluted to fifty times its volume, 0.006744 divided by 50, or 0.00013488 cubic millimeter, would be the volume of the original milk contained in the standard tube. In the case of cream where it is diluted to one hundred times its volume one-half this figure represents the volume of original cream in the standard tube. To find the number of globules present in 0.0001 cubic millimeter it is only necessary to

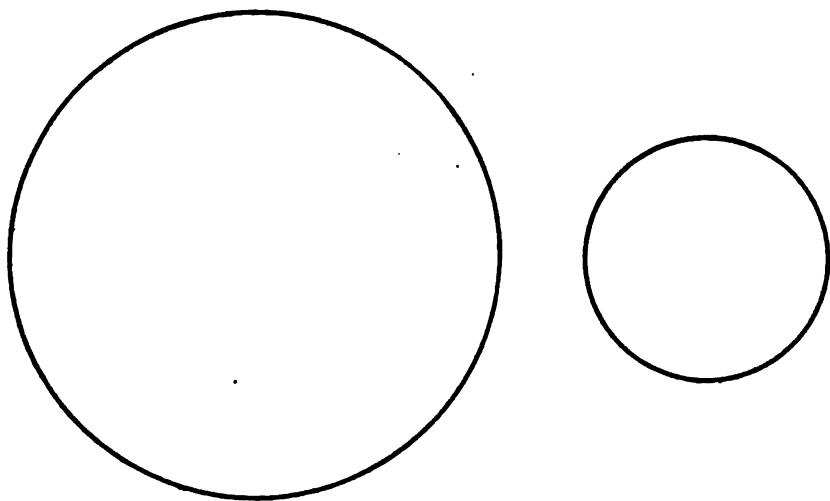


FIG. 2.—Showing the average relative size of large and small fat globules.

multiply the number in the standard tube by the factor 0.7414, obtained by dividing 0.0001 by 0.00013488.

The relative size is found by dividing the percentage of fat by the number of globules in 0.0001 cubic millimeter of the milk or cream. To avoid fractions, the figure obtained in this way is multiplied by 10,000.

An example illustrating the method of calculating may be given. A sample of milk containing 4 per cent of fat is chosen. Let 30 represent the number of globules counted in 50 divisions of a tube whose

^a The volume of two cylinders having the same height but different diameters are to each other as the squares of their diameters. If we let n equal the number of globules in the observed tube, n'' the number in the standard tube, d the diameter of the observed tube, and 100 the diameter of the standard tube, then $n:n'' : d : 100$, or n'' equals $\frac{10,000 n}{d^2}$.

internal diameter has been found to be 40 divisions. Applying the formula $\frac{10,000 n}{d^3}$, we have $\frac{300,000}{1,600} = 187$, or the number of globules which would be found in a standard tube whose length is 50 divisions and whose diameter is 100 divisions. Multiplying 187 by the factor 0.7414, we have 138, which would be the number of globules in 0.0001 cubic millimeter. Dividing 4 by 138 and multiplying by 10,000, we have 290 as the relative size of the fat globules in the example taken. As Doctor Babcock states, the relative size is not strictly accurate, since percentages by weight have been confused with volume. To be strictly accurate we must multiply the relative size by a factor obtained by dividing the specific gravity of the milk by the specific gravity of the butterfat. However, since relative values only are required in our work, it was decided that the relative size would answer the purpose, so our calculations have stopped at that point and the results are expressed in terms of relative size.

RESULTS OF EXPERIMENTS.

The following tables present data as to source of samples and results of the experiments:

TABLE 1.—Data concerning source of samples.

Sample No.	Date.	Breed of cow.	Date of last calving.	Time in milk.	Age of cow.	Daily yield of milk when sample was taken.	Average fat content.
	1906		1906.	Days.	Yrs.Mos.	Pounds.	Per cent.
1	Sept. 25	Ayrshire	July 10	77	2 5	21.5	4.0
		do.	Aug. 13	43	3 8	28.9	3.9
		do.	July 29	58	3 2	25.1	4.0
2	Oct. 3	Jersey	May 8	151	9 2	27.9	4.5
		do.	Aug. 19	45	9 1	30.3	4.1
		do.	May 8	148	9 0	22.1	5.0
3	Oct. 9	do.	Dec. 15	298	8 11	10.3	5.6
		do.	Jan. 26	256	2 7	11.1	5.2
		do.	Feb. 7	244	3 3	10.3	5.8
4	Oct. 28	do.	Dec. 6	307	8 11	7.4	6.7
		do.	Jan. 25	257	4 7	7.5	5.5
		do.	Feb. 6	345	9 0	9.1	5.0
4	Oct. 28	Holstein	Apr. 18	193	6 4	32.2	3.2
		do.	Apr. 29	182	4 6	20.6	3.0
		do.	May 13	168	4 5	31.9	3.4
		do.	Feb. 11	259	4 4	23.1	3.0
	1908.		1907.				
5	Jan. 17	Shorthorn	Sept. 30	109	4 8	19.7	3.9
		do.	Oct. 13	96	6 3	18.9	4.1
6	Jan. 20	do.	Sept. 30	112	4 8	20.5	3.9
		do.	Oct. 13	99	6 3	19.2	4.1
7	Jan. 23	Ayrshire	Dec. 27	27	3 9	31.9	3.9
		do.	Sept. 27	118	5 1	22.1	3.75
8	Jan. 26	Holstein	July 17	192	5 10	24.2	3.4
		do.	May 31	240	5 9	24.9	3.3
9	Feb. 11	do.	July 20	190	4 2	23.8	3.2
		do.	Aug. 13	182	7 8	31.2	3.1
10	Feb. 21	do.	May 7	279	5 5	26.3	3.0
		Jersey	June 16	250	6 6	18.5	6.48
		do.	June 12	254	4 5	26.1	5.86

TABLE 2.—*Results of microscopical work.*

Sample No.	Size of globules.	Relative size of globules.	Percentage of globules less than one division in diameter.	Percentage of globules between one and two divisions in diameter.	Percentage of globules over two divisions in diameter.
1	Small	161			
1	Large	631			
2	Small	91.6			
2	Large	741			
3	Small	112			
3	Large	698			
4	Small	51.7			
4	Large	316			
5	Small	21.7	54.2	40.4	1.3
5	Large	590	15.6	38.4	46.0
6	Small	66.8	45.9	49.3	4.8
6	Large	530	13.8	38.3	46.9
7	Small	12.9	73.2	26.6	0.2
7	Large	703	31.6	61.1	7.3
8	Small	44.2	67.8	41.9	0.3
8	Large	417	19.3	69.9	10.8
9	Small	42.3	61.2	38.5	0.3
9	Large	299	14.6	80.0	5.4
10	Small	63.5	67.0	32.3	0.7
10	Large	691	7.3	70.9	21.8

TABLE 3.—*Results of chemical work.*

Sample No.	Size of globules.	Specific gravity.	Melting point.	Refractive index.	Koettstorfer number.	Reichert-Meissl number.	Iodin number.	Hiehner number.
1	Small			1.4609	231.2	27.29	30.91	
1	Large			1.4609	230.6	27.36	30.55	
2	Small			1.4608	229.8	27.36	31.33	
2	Large			1.4606	230.6	28.13	32.29	
3	Small			1.4615	234.0	25.69	32.86	
3	Large			1.4613	234.6	25.49	32.83	
4	Small			1.4614	228.3	25.69	33.37	
4	Large			1.4610	228.0	26.28	33.45	
5	Small	0.9078	33.47	1.4565				
5	Large	0.9044	33.33	1.4583	232.4	29.73	29.73	87.20
6	Small	0.9038	33.50	1.4564	231.0		29.72	86.83
6	Large	0.9033	33.38	1.4562	233.3	24.15	30.20	86.83
7	Small	0.9038	33.37	1.4567	229.6	25.01	30.54	86.24
7	Large	0.9035	33.37	1.4568	228.5	26.06	31.25	87.16
8	Small	0.9053	32.37	1.4575	226.6	24.03	34.56	88.53
8	Large	0.9044	32.90	1.4574	226.3	24.20	34.39	88.35
9	Small	0.9014	31.85	1.4575	227.6	24.98	35.42	85.73
9	Large	0.9014	32.43	1.4573	227.9	25.90	35.44	85.50
10	Small	0.9069	32.43	1.4561	233.8	26.93	28.25	86.13
10	Large	0.9065	32.90	1.4560	234.9	27.63	27.62	86.83

a Sample too small for complete analysis.

TABLE 4.—*Color of butter and butterfat from large and small globules.*

Sample No.	Size of globules.	Butter.		Butterfat.	
		Yellow.	Red.	Yellow.	Red.
5	Small			15.5	1.5
5	Large			15.5	1.5
6	Small			15.5	1.5
6	Large			15.5	1.7
7	Small			27.2	1.1
7	Large	0.9	0.9	27.2	1.6
8	Small	.8	.8	20.0	1.5
8	Large	.8	.8	20.0	1.5
9	Small	.7	.8	11.0	.5
9	Large	.7	.7	10.7	.3
10	Small	1.0	.8	22.0	1.2
10	Large	.9	.9	21.0	1.0

CONCLUSION.

It is impossible by any known process to effect a complete separation of the large and the small fat globules in milk. The best that can be expected is to secure two creams from the milk, one of which contains a large percentage of large globules and the other a large percentage of small globules, or, in other words, one in which the relative size of the fat globules is comparatively large and another in which it is comparatively small. That such has been obtained in this investigation will be shown by Table 2. A study of Table 3 will fail to reveal any considerable differences between the fat from the large-globule cream and that from the small-globule cream. The differences in all cases are small and in most cases well within the limits of legitimate experimental error. Table 4 also shows little or no variation in color.

The investigation, of course, could be extended by introducing more animals and more samples, but the range covered by the animals selected and the number of determinations made would seem to be sufficient to warrant the conclusion that in homogeneous milk the large and small fat globules have identical chemical and physical composition.

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Issued June 25, 1909.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY.—BULLETIN 112.
A. D. MELVIN, CHIEF OF BUREAU.

THE LOCO-WEED DISEASE OF THE PLAINS.

BY

C. DWIGHT MARSH,
*Expert, Poisonous-Plant Investigations,
Bureau of Plant Industry.*



WASHINGTON:
GOVERNMENT PRINTING OFFICE.
1909.

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LETTER OF TRANSMITTAL.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY,
Washington, D. C., January 16, 1909.

SIR: I have the honor to transmit herewith, for publication as a bulletin of this Bureau, a manuscript entitled "The Loco-Weed Disease of the Plains," by Dr. C. Dwight Marsh, expert, of the Office of Poisonous Plant Investigations, Bureau of Plant Industry, being a report of field investigations carried on during 1905, 1906, and 1907.

Although the work reported in this paper was done under the supervision of the Bureau of Plant Industry, it has been considered desirable, inasmuch as it deals largely with subjects coming within the scope of the Bureau of Animal Industry, such as the symptomatology, pathology, and treatment of a disease of animals, to have it published as a bulletin of the latter Bureau.

For many years the so-called loco disease has been a cause of heavy loss to the stockmen of the West, and it has been generally attributed by them to certain plants eaten by the stock. As far back as 1886 the Bureau of Animal Industry investigated the subject, and an article by Dr. M. Stalker in the Third Annual Report of the Bureau has up to the present time remained the most satisfactory published description of the disease. While Doctor Stalker in that report expressed the belief that the loco plant "is possessed of some toxic property that has a specific effect on the nerve centers," and while these plants have long been classed as poisonous in the publications of this Department, it has remained for Doctor Marsh in the work herein reported to present experimental proof that the plants have a poisonous effect and really cause the disease and to suggest a line of treatment, and for Dr. A. C. Crawford, in Bulletin 129 of the Bureau of Plant Industry, to discover and identify barium as a poisonous element in the plants.

Respectfully,

A. D. MELVIN,
Chief of Bureau.

HON. JAMES WILSON,
Secretary of Agriculture.

LETTER OF SUBMITTAL.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF PLANT INDUSTRY,
Washington, D. C., July 10, 1908.

SIR: I take pleasure in handing you herewith, for publication as a bulletin of the Bureau of Animal Industry, the accompanying manuscript presenting a technical report entitled "The Loco-Weed Disease of the Plains," prepared by Dr. C. Dwight Marsh, expert, working under the direction of Dr. Rodney H. True, Physiologist in Charge of Poisonous-Plant Investigations in the Bureau of Plant Industry.

This bulletin presents the evidence obtained from an experimental field study of the relation of the so-called "loco weeds" to the losses frequently attributed to the eating of them by horses, cattle, and sheep. The plants studied are those characteristic of the plains, and work has been carried on under actual field and corral conditions. The investigation has been fully successful in proving the suspected plants to be the cause of great and widely scattered stock losses. It has made possible a satisfactory description of the disease and furnishes a basis on which other disorders, now confused with loco-weed disease, but of different origin, may be distinguished from it. It has developed a method of successfully treating a large percentage of loco cases when such occur under circumstances making the frequent handling of the animals practicable.

A companion bulletin, dealing with the technical laboratory studies carried on at the same time in Washington by Dr. A. C. Crawford, pharmacologist, is a necessary supplement to this bulletin for anyone desiring a full account of the loco-weed disease. This bulletin is entitled "Barium, a Cause of Loco-Weed Poisoning," and constitutes Bulletin No. 129 of the series of this Bureau.

Respectfully,

B. T. GALLOWAY,
Chief of Bureau.

Dr. A. D. MELVIN,
Chief, Bureau of Animal Industry.

P R E F A C E .

The so-called "loco-weed disease" of horses, sheep, and cattle has been a source of most serious complaint for many years, especially from such stockmen as have grazed their animals on the Great Plains east of the Rocky Mountains. While the losses have varied in severity from year to year, they have reached such a magnitude as to make the matter one of national concern. On account of both the economic importance of the problem and of its intrinsic scientific interest numerous investigations approaching the subject from the most varied standpoints have been undertaken. As a net result there has appeared a large body of literature marked by a wide diversity in the conclusions reached. This discouraging state of disagreement prevailed among the reported results of the field studies on the subject prior to the time at which the Office of Poisonous Plant Investigations undertook its campaign, but it was believed that by laying siege to the problem in a more thoroughgoing and persistent way than any of its predecessors had been in a position to do it might still be able to ascertain the essential facts of the loco situation.

Accordingly, in 1905 a field station was established at Hugo, Colo., in cooperation with the Colorado Agricultural Experiment Station, which furnished the animals used, and experimental feeding work on horses and cattle was begun. The state of opinion both among practical stockmen and among scientists had made clear the first step to be taken. Popular opinion had long pointed to the eating of certain members of the pea family (Leguminosæ), especially *Astragalus mollissimus*, the purple loco weed, and to *Aragallus lamberti*, the rattleweed, as the probable cause of poisoning. The first task was to test the accuracy of this report. Accordingly feeding experiments under field and corral conditions on plants of known origin were undertaken at Hugo, and parallel laboratory tests were carried on at Washington. The results showed clearly that loco symptoms were produced as a result of loco-weed feeding, and as the investigation proceeded the characteristics of this chronic, progressive disease were thoroughly worked out.

The disagreement above referred to arose from a number of causes, not least of which was the kind of animal upon which the investigations had been carried out. Those working on sheep had seen much reason to regard the symptoms observed as fully accounted for by the various kinds of animal parasites with which this animal

is affected in regions in which the loco disease has been regarded as prevalent. Another reason for disagreement lay in the fact that the characteristic symptoms of the loco-weed disease had not been sharply presented as a result of the experimental production of the disease; hence "loco" has been a general term covering many kinds of trouble of a more or less chronic type. Indeed, there seems to be good reason for believing that in some cases starvation has been called "loco." Another source of confusion has lain in the fact that investigators taking a more or less superficial view of the matter had seen animals in various stages of the disorder and had not gone into the matter deeply enough to correlate the different phases of a progressive disease.

The second stage of the field work looked forward to measures of relief. The behavior of the plants concerned was looked into and methods of eradication tried. Contrary to frequently expressed belief, these weeds were found to be easy of extirpation by thoroughly digging them out. Methods of meeting the trouble from the standpoint of the animals concerned were in a degree worked out.

Laboratory work carried on at Washington seemed to show that the loco plants collected in the vicinity of the Hugo station—and doubtless those of eastern Colorado, Nebraska, and Kansas—owe their poisonous character, to a certain degree, to the presence in them of barium absorbed from the soil. For a fair understanding of the loco situation both the bulletin herewith presented, dealing with the field phase of the work, and the companion publication on "Barium, a Cause of the Loco-Weed Disease," based on the laboratory work, must necessarily be taken into account. Unfortunately the nature of these conclusions was not indicated until near the close of the work for the season of 1907, so that the work in the direction of remedial measures gained no advantage from this information. Those measures which were found to be most beneficial are shown, however, by the light from the laboratory result, to be well founded. The field work has been carried out by Dr. C. Dwight Marsh, expert, and his assistants, the laboratory work by Dr. A. C. Crawford, pharmacologist.

The present work by Doctor Marsh consists of a technical report on prolonged field feeding investigations in connection with the loco problem, carried out at Hugo and Woodland Park, Colo., and Imperial, Nebr., in cooperation with the Colorado Agricultural Experiment Station and the Nebraska Agricultural Experiment Station. Doctor Marsh has here succeeded in working out very completely and more thoroughly than has ever been done heretofore the symptomatology of the true loco disease, and has given a basis on which to distinguish the trouble known as loco, properly speaking, from various types of pseudo-loco which have gone under this name. He has also described the plants which cause the trouble and outlined their distribution.

The experiments intended to secure the extermination of the plants are summarized, the conclusion of which is that the most practicable way of disposing of the plants is to dig them out. This can be done on land which is taken up by settlers, and will relieve a large number of sufferers. The question of the open range, in so far as it is still Government property, is one which we see no way of handling except by Federal appropriation.

The treatment of animals is also outlined, it appearing that horses and cattle require somewhat different handling. Horses are helped by daily treatment with Fowler's solution, and cattle do best with slight daily doses of strychnin, accompanied with laxative feed and occasional doses of magnesium sulphate. Further work is planned on wholesale antidoting under range conditions.

R. H. TRUE,
*Physiologist in Charge, Poisonous-Plant Investigations,
Bureau of Plant Industry.*

CONTENTS.

	Page.
PART I.—INTRODUCTION.....	15
Historical summary and review of literature.....	15
Symptoms mentioned in literature.....	32
Remedies proposed.....	33
Conclusions from literature.....	34
Plants known as loco plants.....	36
List of species.....	36
Description of <i>Aragallus lamberti</i>	37
Description of <i>Astragalus mollissimus</i>	38
Dispersal of <i>Aragallus lamberti</i> and <i>Astragalus mollissimus</i>	39
Relations of loco plants to fertile soil.....	40
Experimental work limited to two plants.....	40
PART II.—EXPERIMENTAL WORK.....	42
The problem to be solved.....	42
Plan of work.....	43
Location of experiment.....	43
Preliminary work.....	44
Work of the first season.....	45
Experiments with cattle.....	47
Experiments with horses.....	49
Cases of cattle and horses not subjects of experiment.....	52
Summary of first season's work.....	54
Work of the second and third seasons.....	55
Experiments with cattle.....	56
Experiments with horses.....	62
Experiments with sheep.....	66
Discussion of sheep experiments.....	69
Experiment at Woodland Park, Colo.....	71
Experiment at Imperial, Nebr.....	73
Remedial measures.....	73
Treatment with strychnin in 1906.....	75
Treatment with arsenic in 1906.....	78
Results of treatment in 1906.....	81
Treatment of cattle in 1907.....	83
Treatment of horses in 1907.....	88
Treatment of sheep in 1907.....	90
PART III.—RESULTS AND CONCLUSIONS.....	91
Cause of the disease.....	91
Symptoms of loco poisoning.....	92
Pathological lesions.....	95
Examination of blood.....	95
Walls of stomach.....	97
Hemolymph glands.....	97
Nervous system.....	97

PART III.—RESULTS AND CONCLUSIONS—Continued.	Page.
Virulence of loco poison.....	98
Time required to produce poisoning.....	98
Amount of loco necessary to produce poisoning.....	100
Relative importance of <i>Astragalus mollissimus</i> and <i>Aragallus lamberti</i>	101
Susceptibility of different breeds.....	102
Age susceptibility.....	102
Effect on animals other than horses, cattle, and sheep.....	103
Effect on man.....	104
Losses from loco poisoning.....	104
Destruction of loco weeds.....	105
Insects destroying loco plants.....	106
Effect of dry seasons.....	108
Remedies.....	108
Treatment of cattle in 1907.....	110
Treatment of horses in 1907.....	110
General results of treatment.....	111
Results with cattle.....	111
Results with horses.....	111
Permanency of loco cures.....	111
Prevention of loco poisoning.....	112
Summary of work on remedies.....	112
GENERAL SUMMARY.....	113
BIBLIOGRAPHY.....	117
INDEX.....	127

ILLUSTRATIONS.

PLATES.

	Page.
PLATE I. White loco, or rattle weed (<i>Aragallus lamberti</i>). Fig. 1.—Plant in flower. Fig. 2.—Plant in fruit.....	38
II. Purple or woolly loco (<i>Astragalus mollissimus</i>). Fig. 1.—Flower and fruit. Fig. 2.—Habit of the plant.....	38
III. Fig. 1.—Case 8, July 19, 1905. Just before the locoed condition was particularly evident. Fig. 2.—Case 8, August 3, 1905. When animal showed typical symptoms of loco poisoning. Fig. 3.—Case 8, August 23, 1905. Emaciated condition of the animal and typical loco attitude of lowered head and braced legs. Fig. 4.—Case 8, August 23, 1905. Loco leaping unnecessarily high in going over a rut in the road. Fig. 5.—Case 8, August 23, 1905. Lifting foot unnecessarily high in passing over a wire. Fig. 6.—Case 8, August 27, 1905. Animal too weak to stand unaided.....	50
IV. Fig. 1.—Case 18, May 12, 1905. A mare weakened by loco poisoning. Fig. 2.—Case 18, June 27, 1905. Advanced condition of loco poisoning. Fig. 3.—Case 19, May 16, 1905. Before eating any of the loco weed. Fig. 4.—Case 19, August 31, 1905. Animal eating loco at a time when it has lost some flesh, but was not in bad condition. Fig. 5.—Case 19, October 15, 1905. Animal in a very much reduced and emaciated condition. Fig. 6.—Case 19, October 26, 1905. Animal just before its death.....	50
V. Fig. 1.—Case 524. Peculiar way in which a locoed horse uses its mouth in attempting to eat. Fig. 2.—Case 533. How a locoed horse will rear when suddenly startled. Fig. 3.—Case 525. Peculiar gait which a badly locoed horse exhibits. Fig. 4.—Case 525. Horse rearing when suddenly startled by a hat thrown out in front of it. Fig. 5.—Case 529. A locoed angora goat unable to get upon its feet, but otherwise fairly well. Fig. 6.—Another attitude of case 529.....	50
VI. Fig. 1.—Case 3, July 15, 1906. Effects of loco poisoning shown in emaciation and dejected attitude just before death of animal. Fig. 2.—Inner wall of fourth stomach of case 3, showing ulcers upon its surface.....	58
VII. Fig. 1.—Case 2, July 25, 1906. Steer bending low to avoid an imaginary obstruction above it while passing through a gate. Fig. 2.—Case 34. Another case similar to that in figure 1. Fig. 3.—Case 67, May 1, 1906. A bright heifer calf shortly after birth. Fig. 4.—Case 67, July 5, 1906. Calf showing effects of loco which it had eaten in imitation of its mother. Fig. 5.—Case 67, October 16, 1906. Calf with typical symptoms of loco. Fig. 6.—Case 67, October 19, 1906. Animal just before death.....	58

- PLATE VIII. Fig. 1.—Case 16, June 6, 1906. Horse before eating loco weed. Fig. 2.—Case 16, September 15, 1906. Animal in an advanced stage of loco poisoning. Fig. 3.—Case 52, May 19, 1906. Mule in fairly good condition before feeding upon loco weed. Fig. 4.—Case 52, September 7, 1906. Last stages of loco poisoning. Fig. 5.—Case 50, July 19, 1906. An old horse before being fed with the loco weed. Fig. 6.—Case 50, September 18, 1906. Effect of loco poisoning in attitude and emaciated condition; also shows cuts produced by running into a barbed-wire fence. . . . 66
- IX. Fig. 1.—A group of locoed sheep. Fig. 2.—Sheep 27. A locoed sheep in the last stages of poisoning. Fig. 3.—Sheep 36. Effect of loco poisoning combined with grub in the head. Fig. 4.—Same as figure 3, different attitude. Fig. 5.—Case 66, October 20, 1906. A locoed lamb. Fig. 6.—Case 66, November 12, 1906. Attitude of the animal just before its death. . . . 66
- X. Fig. 1.—Case 4, May 30, 1905. Before feeding on loco weed. Fig. 2.—Case 4, September 7, 1906. Showing typical symptoms of loco poisoning. Fig. 3.—Case 4, September 15, 1906. Animal under influence of loco and with a noticeable accumulation of serous fluid under its chin. Fig. 4.—Case 4, August 22, 1907. Same animal in succeeding year, when it no longer showed any symptoms of loco poisoning. Fig. 5.—Case 536, July 9, 1906. A locoed steer with curvature of the fetlock joints. Fig. 6.—Case 536, July 25, 1906. The same animal after treatment with strychnin. . . . 76
- XI. Fig. 1.—Case 547, April 23, 1907. A heifer in an advanced stage of loco poisoning. Fig. 2.—Case 547, August 22, 1907. Completely recovered under treatment. Fig. 3.—Case 551, June 14, 1907. A horse in the advanced stages of loco poisoning. Fig. 4.—Case 551, September 20, 1907. After course of treatment. Fig. 5.—Case 71, June 10, 1907. A locoed sheep, emaciated and extremely nervous. Fig. 6.—Case 71, September 22, 1907. Recovered, as the result of the course of treatment. . . . 88

TEXT FIGURES.

FIG. 1. Distribution of <i>Aragallus lamberti</i> in the United States.	37
2. Distribution of <i>Astragalus mollissimus</i> in the United States.	39
3. Case 513. A locoed colt stunted in its growth by loco poisoning.	44
4. Curve of weight of steer No. 10, 1906.	58
5. Curve of weight of steer No. 10, 1907.	59
6. Curve of weight of cow No. 32.	59
7. Curve of weight of cow No. 37.	60
8. Curve of weight of calf No. 66.	61
9. Curve of weight of calf No. 67.	62
10. Curve of weight of calf No. 68.	63
11. Curve of weight of horse No. 16.	63
12. Curve of weight of horse No. 50.	64
13. Curve of weight of horse No. 52.	65
14. Curve of weight of horse No. 59.	66
15. Curve of weight of steer No. 4, 1906.	76
16. Curve of weight of steer No. 4, 1907.	76
17. Curve of weight of steer No. 40.	78
18. Curve of weight of horse No. 58.	79

	Page
FIG. 19. Case 28, August 27, 1906. Locoed cow, but not a bad case	80
20. Curve of weight of cow No. 28, 1906	81
21. Curve of weight of cow No. 28, 1907	82
22. Curve of weight of steer No. 42, 1906	84
23. Curve of weight of steer No. 42, 1907	84
24. Curve of weight of cow No. 35	85
25. Curve of weight of heifer No. 548	85
26. Curve of weight of steer No. 38	87
27. Curve of weight of heifer No. 547	87
28. Curve of weight of steer No. 555	88
29. Curve of weight of horse No. 551	89

THE LOCO-WEED DISEASE OF THE PLAINS.

PART I.—INTRODUCTION.

HISTORICAL SUMMARY AND REVIEW OF LITERATURE.

The word "loco" is of Spanish origin, in which language it is used both as a noun and as an adjective, meaning crazy. It has been popularly applied to a considerable number of plants in the semiarid region of the Western and Southwestern States because of the effects these plants are supposed to produce on animals.

The first published account of the loco plants was by Doctor Vasey in the monthly report of the Commissioner of Agriculture for October, 1873. He quotes a letter from O. B. Ormsby, of Bakersfield, Cal., who describes the effect of the plant in the following terms:

It prevails quite abundantly over an extent of 150 square miles in this valley, and, I am informed, is found in other valleys of the State, and also in Arizona. This year the army-worm, and a minute insect which destroys the seeds, have killed a great deal of it, but if not molested it will soon flourish to as great an extent as ever. I think very few, if any, animals eat the loco at first from choice; but, as it resists the drought until other feed is scarce, they are at first starved to it, and after eating it a short time appear to prefer it to anything else. Cows are poisoned by it as well as horses, but it takes more of it to affect them. It is also said to poison sheep. As I have seen its action on the horse, the first symptom of the poisoning, apparently, is hallucination. When led or ridden up to some little obstruction, such as a bar or rail lying in the road, he stops short, and, if urged, leaps as though it were four feet high. Next he is seized with fits of mania, in which he is quite uncontrollable, and sometimes dangerous. He rears, sometimes even falling backward, runs or gives several successive leaps forward, and generally falls. His eyes are rolled upward until only the white can be seen, which is strongly injected, and, as he sees nothing, he is as apt to leap against a wall or man as in any other direction. Anything which excites him appears to induce the fits, which, I think are more apt to occur in crossing water than elsewhere, and the animal sometimes falls so exhausted as to drown in water not over 2 feet deep. He loses flesh from the first, and sometimes presents the appearance of a walking skeleton. In the next and last stage, he only goes from the "loco" to water and back, his gait is feeble and uncertain, his eyes are sunken, and have a flat, glassy look, and his coat is rough and lusterless. In general, the animal appears to perish from starvation and constant excitement of the nervous system, but sometimes appears to suffer acute pain, causing him to expend his strength in running wildly from place to place, pawing and rolling, until he falls, and dies in a few minutes.

The specimen sent, Doctor Vasey said, was an *Astragalus*. In the report of the Commissioner of Agriculture for 1874 Doctor Vasey quotes this same letter of Ormsby and refers to other reports received from California. The plants sent to him were determined as *As-*

tragalus hornii Gr. and *Astragalus lentiginosus* Gr. He states, too, in this report that Doctor Moffatt, of the United States Army, had sent in specimens of *Oxytropis lamberti*, from Colorado, with the following description of its effects:

Cattlemen inform me that a weed grows among the grass, particularly in damp ground, which is poisonous to horned cattle and horses and destroys many of them. From the manner in which they describe its effects upon the animals it must be of the nature of a narcotic, and they assure me that cattle, after having eaten it, may linger many months or for a year or two, but invariably die at last from the effects of it. The animal does not lose in flesh apparently, but totters on its limbs and becomes crazy. While in this condition, a cow may lose her calf and never find it again and will not recognize it if presented to her. The sight becomes affected, so that the animal has no knowledge of distance, but will make an effort to step or jump over a stream or an obstacle while at a distance off and will plunge into it or walk up against it upon arriving at it. The plant was pointed out to me, and seems to be related to the lupin.

In 1875 Mr. Kellogg, in an article on California and Colorado loco poisons, in the Proceedings of the Academy of Sciences of California, states that thousands of horses, cattle, and sheep have been poisoned by *Astragalus menziesii* in California. He gives a description also of *Oxytropis lamberti*.

Professor Prescott in 1878 gave an account of the attempts of Miss Watson to extract a poisonous principle from *Oxytropis lamberti*. He states that Mr. Birdsall tried some of the ground root on himself, but with little effect.

In an article in the American Journal of Pharmacy in 1879 Maisch adds *Astragalus mollissimus* to the list of loco plants, and says that he is informed that horses dig in the earth for it, become intoxicated, seek water, drink with avidity, swell up, fall over, and die.

In the report of the Commissioner of Agriculture for 1878 Professor Collier refers to specimens of *Oxytropis lamberti*, which he says were received for examination. He makes a brief reference to the work of Miss Watson.

In 1880 Professor Collier, in the report of the chemist to the Commissioner of Agriculture for 1879, makes a preliminary report on *Astragalus mollissimus* and *Oxytropis lamberti*. He states that as a result of the analyses the only substances likely to be poisonous in *Astragalus mollissimus* are an alkaloid and a bitter extractive; the harm being done by *Oxytropis lamberti* probably, by the fact that the sweet taste of the plant leads the animals to eat a substance which is mechanically unfit for food, but it is possible that if the animal becomes enfeebled the small amount of alkaloids present may produce poisonous effects.

Ott, in 1882, in an article in "New Remedies" states that the loco plants are *Oxytropis lamberti*, *Astragalus mollissimus*, and *Sophora sericea*. These may prove fatal in two or three days; other

cases linger a year or more. Horses are more affected than cattle or sheep. When first eaten an exhilarating effect is produced which tempts the animal to eat more. Later the animal contracts the habit. It does not acquire the habit in summer when there is an abundance of grass. He then states that the effects are as follows:

1. A small amount of exercise will produce profuse sweating.
2. The animal when "shying" at an object is very liable to rear up and fall backward.
3. A stroke, or even a touch, about the head seems to rouse the disorder, which is immediately manifested by struggles and general frenzy.
4. While being ridden, when that is possible, the animal steps very high, as though walking over obstacles, and the slightest thing in the road, as a stick, piece of rope, or wagon rut, will be magnified into an apparently insurmountable difficulty, and it is ludicrous to see the animal jump as though leaping over a ditch in an effort to get over it.
5. The animal loses all power of reasoning or of coordination, will walk into a ditch or bank instead of around it, and will sometimes walk backward when apparently trying to go forward.

Ott made an alcoholic extract of *Astragalus mollissimus* and treated subcutaneously frogs, rabbits, and a cat. The rabbits were used for circulation tests. He summarizes the results of his pharmacological tests as follows:

1. It decreases the irritability of the motor nerves.
2. It greatly affects the sensory ganglia of the central nervous system, preventing them from readily receiving impressions.
3. It has a spinal tetanic action.
4. It kills mainly by arrest of the heart.
5. It increases the salivary secretion.
6. It has a stupefying action on the brain.
7. It reduces the cardiac force and frequency.
8. It temporarily increases arterial tension, but finally decreases it.
9. It greatly dilates the pupil.

In 1885 Mohr gives the distribution of the species, stating that they are found in New Mexico, Colorado, Utah, Nevada, Arizona, and California. He gives the following list of species as loco plants: *Astragalus mortoni*, *A. hornii*, *A. lentiginosus* var. *fremontii*, *A. cocarpa* (*oocarpa* probably intended), *A. crotolarix*, and *Oxytropis lamberti*.

Up to this time all reports agree as to the poisonous character of the plants, but an anonymous author in the American Veterinary Review of 1885 states positively that the plant is not poisonous. He says that when cattle eat a quantity it absorbs the juices of the alimentary canal and dries them up. It then collects in the stomach and intestines as a dry mass, and the pressure on the circulation causes all the symptoms observable and finally produces death. He declares that the internal condition of cattle with this disease verifies this view.

Havard in 1885 says that *Astragalus mollissimus* is the loco plant of western Texas. Animals avoid it, and eat it only through inadvertence or necessity. They lose appetite, become stupid and thin, have tremors, and lose coordination. *Oxytropis lamberti* is found only in northern Texas, and is nowhere common.

Hurd in 1885 states that the loco plants in California are *Astragalus crotolaria*, *A. lentiginosus*, and *Oxytropis lamberti*. He says, too, that animals eat loco only when driven by hunger, but they soon crave it and wander miles in search of it. They become intoxicated, can not be led through a gate, walk mincingly, shy, have an unsteady gait, and become ataxic; when driven into water they may lie down and refuse to rise. They become emaciated and finally die of exhaustion. Complete recovery never occurs.

In the Cornhill Magazine of 1886 there is an article entitled, "The cowboy at home," in which is a somewhat detailed description of the phenomena of loco poisoning in horses, as follows:

We passed a heap of about 40 dead horses piled up together, the most revolting, pitiful spectacle imaginable, and standing listlessly, or moving about with staggering gait, were about twice as many more still living creatures with a peculiar look and manner about them such as I had never seen before, and most of whom must surely soon be dragged to the big heap close by. But what on earth did it all mean? It seemed so strange suddenly to come across this gruesome sight on the lone prairie. I soon learned the explanation.

A weed called "loco" has of late years largely increased in some of the cattle ranges of Texas and the Indian Territory, owing, probably, to an increase in the rainfall. It has a mysterious habit of appearing suddenly in places where it was before unknown, and, given a dry season, of as suddenly disappearing. During the summer, when the prairie is a fair expanse of waving grass, lit up with bright flowers, both horses and cattle instinctively avoid it, but when in the fall of the year the grass becomes scarce in overstocked regions, and when all around assumes a brown and burnt-up appearance, it stands out conspicuously and temptingly green, its long, soft, velvety leaves rising in a bunch from 6 inches to a foot off the ground. Then the hungry creatures begin by nibbling, suspiciously and stealthily, at the seductive plant, but very soon become reckless, and eagerly and greedily devour all that comes in their way. And now, if the mania can not be nipped in the bud by a sufficiency of good strong food, the animal is doomed, for he has become a confirmed "loco-eater." He will rapidly become thin and lose all control over his movements; he will be subject to frequent fits, during which he lies on the ground groaning and foaming at the mouth; he throws himself about without reason; rears up or runs about in small circles when you attempt to mount him; his eyes turn dull and stupid; in short, he gives you the impression of being bereft of his senses. Specimens of loco have been subjected to analysis by experts in Washington and in Edinburgh, but without anything injurious being discovered in it. It is possible that some minute animalculæ may be the cause of the mischief, but up to the present its disastrous effects only are known, for this pernicious weed causes periodically the death of thousands of horses and cattle.

Professor Sayre, of the University of Kansas, in 1886 published in the Transactions of the Kansas Academy of Sciences a somewhat extended account of loco and locoed animals. He visited New Mexico, Colorado, and Kansas, and states that the plant appears at

Medicine Lodge and extends southwesterly into the Indian Territory and northwesterly through Kansas. He says that a veterinary surgeon, Doctor Harding, took two horses with a taste for loco, and kept one in a pasture with loco and the other in a loco-free pasture, with the result that the one died and the other recovered. Two others were fed, one with hay and the other with dead loco. The first one lived and the second died. He gives a description of the symptoms of locoed animals, the result of his personal inquiry among ranchmen and the report of a Mr. R. E. Steele. It is somewhat interesting, however, that many parts of this description agree word by word, and sentence by sentence, with a letter of Mr. Ormsby quoted in Vasey's report of 1873.

Sayre states that the loco disease is due to two plants, *Astragalus mollissimus* and *Oxytropis lamberti*. He made a preliminary chemical examination which was apparently never completed. He also held an autopsy on a locoed cow belonging to Mr. Steele. The cow was undersized, poor, stupid, unsteady, with short breathing and shaking head. He reports that the reticulum and psalterium were softened. The tissues through the intestines were degenerated; the peritoneum and the omentum were inflamed with tumors the size of a pea; the heart was one-third larger than normal, with the mitral and tricuspid valves inflamed; the bile was thin and watery, and the inner coat of the bladder softened; the membranes of the brain were congested and adherent, the brain was paler than normal, and the membranes of the spinal cord were inflamed and adherent. He concludes that the disease is one of the mucous and serous membranes.

In the same year Professor Sayre published an article in the report of the Kansas State board of agriculture, which was, to a large extent, a repetition of the material published in the Transactions of the Kansas Academy of Sciences. He adds, however, a report that the plant causes abortion in cows. He states that as the result of his own chemical examination it would appear that there is no poisonous principle in the plant, and suggests that the hairs upon the leaves may be irritating.

In the Third Annual Report of the Bureau of Animal Industry for 1886 Doctor Stalker gives a report of a personal investigation of the loco disease. He visited the country from western Nebraska to Salt Lake, and from Cheyenne to El Paso. He describes the appearance and habits of the two plants, *Oxytropis lamberti* and *Astragalus mollissimus*. He states that the term loco is used simply to designate a certain disease, as well as being a term applied to plants.

He found that the loco habit is an acquired one and that animals will forget it when kept on loco-free food. All confirmed loco-eaters become complete physical wrecks, showing general derangement of the nervous system and more or less digestive disturbance. In

post-mortem examinations of horses he found a serous effusion in the lateral ventricles, hemorrhagic clots in the fourth ventricle, and sometimes a serous effusion in the arachnoid space. The liver was dense in structure. Immense numbers of bots were found, especially in the duodenum, and he suggests that while these parasites would not account for the clinical symptoms, they may have something to do with the abnormal appetite. In post-mortems on sheep large numbers of tapeworms were found, and these he suggests may account in part for the loco appetite in sheep. He presumes that the plant is possessed of some toxic property that has a specific effect on the nerve centers, and that these effects have a marked tendency to remain permanent, though he was not aware that the poison, if present, had ever been separated by analysis. He states that the disease may be cured by taking animals from the range and feeding them on nutritious diet. He also states that special attention should be given to the destruction of intestinal parasites, and expresses his belief that thousands of supposed cases of loco poisoning are the result alone of such parasites.

In 1887 Behr, in an article on the poisonous plants indigenous to California, states that the notorious loco weed is an *Astragalus*, but that the exact species is not known with certainty. He says that dogs, cats, and rabbits do not suffer if the seeds or herbs are mixed with their food, and that cattle, sheep, and horses suffer only at certain seasons. Poisoning may be due either to infection by some fungoid parasite, perhaps of the *Claviceps* order, or by a substance produced by fermentation or putrefaction, or it is possible that the poison may not be of vegetable origin at all, but caused by some parasite infesting the suspected plant.

Carhart, in the *New York Medical Record* in 1887, speaks of his acquaintance with the effects of loco in the Panhandle. Cattle occasionally eat it, but are rarely affected by it; mules do not suffer from it; but cow ponies die by the thousand. If the taste is acquired they will leave green grass to hunt for it. They break down rapidly, become weak, staggering, foolish, and crazy. A tap on the head will cause them to stagger, rear, and fall backward. Vision is impaired, and there is a loss of muscular coordination. The Indians say that it is due to an insect, but the author has found none. Carhart drank a decoction of loco without effect, and administered the decoction to a locoed horse with apparent good results.

In the *Homeopathic Recorder* in 1887 Doctor Gee gives a somewhat detailed account of some experiments made by some of his students who took doses of loco extract. There seems to be no uniformity in the reports that these students made. They tell of an infinite number of symptoms, many of which, apparently, must have been imaginary.

Dr. T. E. Wilcox, of the United States Army, writes to the Medical Record in 1887 on the subject of loco, and states that the treatment employed by the cowboys in Idaho consists in the amputation of the tails of the animals affected. They claim that they seldom lose any stock when this treatment is instituted early enough. The writer adds that paralysis is probably due to congestion of the spinal cord. It affects the posterior members first. The pupils are dark, as after the use of eserine. *Oxytropis lamberti*, *Astragalus mollissimus*, and possibly others of the Leguminosæ are charged with producing loco poisoning. In Idaho the cowboys call these plants larkspur, although true larkspur is rarely found in the line of march and at that season of the year.

Kingsley in 1888 refers to Kennedy's work, which was at that time unpublished, and gives cases of locoed mules, with report of a post-mortem. In a second letter he concludes that the mules suffered from colic, and this, from his report of the symptoms and the post-mortem, would seem to be probable. He concludes that the loco plant is not poisonous and that the symptoms and pathology are such as would result from overfeeding with any green feed.

Kennedy in 1888, after a description of the plant, tells of his attempts at analysis. He discovered no alkaloid and separated no poisonous principle. He experimented with infusions in boiling water, with dried powder, and with an organic acid separated from the plant. He fed these materials to a dog and there was no effect. He concludes that the plant is not poisonous and that ill effects, if any, are produced by the tough, fibrous, indigestible character of the plant acting as a foreign body, or else its action is identical with an overload of any kind.

The St. Louis Medical and Surgical Journal in an editorial article a little later refers to Kennedy's work, and criticizes him for trying physiological experiments on a dog rather than on an herbivorous animal, and considers his results of no value.

Klench in 1888 gives a general description of symptoms, referring to the papers of Sayre and Stalker, and advises as remedies the administration of potassium bromid or belladonna and aloes, and bleeding.

In 1888 Miller writes of the geographical distribution of *Oxytropis lamberti* and *Astragalus mollissimus* and gives a description of the plants. His description of the effect of the weeds, as well as his remarks in regard to the distribution, are apparently derived from Sayre's publications. He makes the suggestion that inasmuch as loco is said to produce abortion it may be that the plant might be used as an emmenagogue.

Sayre in 1888, in a paper published in the Kansas State board of agriculture report, adds to what he has written in preceding papers a summary of the results of Kennedy and of Ott, and states that he

has tried the extract on himself, but with no result. In the same year, in the Proceedings of the Kansas Pharmaceutical Association, he reports what he has written before and seriously questions the existence of poisonous properties in the plants, considering that the effects may be due to malnutrition, brought about in part by feeding on this weed.

Schwarzkopff in 1888 published his experience with locoed animals in Texas, detailing the symptoms in very much the same terms as those used by previous authors. He used as a remedy atropin and morphia sulphate applied hypodermically. The horse was quiet and acted sensibly in six days. He reports a post-mortem on another horse. He found the cranial sinuses filled with a straw-colored fluid, the vessels of the pia mater injected, and the gray substance reddened and edematous. On the base of the brain inside the arachnoid membrane he found a teaspoonful of pinkish fluid. The medulla and cord were edematous and moist on cut surface.

In 1889 McEachran reports an autopsy near Livermore, Colo. He watched 100 cases of locoed sheep at Givens Ranch, near Colorado Springs, and made post-mortem examinations of two, no details of which are published with the report.

In 1889 Anderson describes the symptoms of locoed animals. He says that the animal has unnaturally bright eyes, froths at the mouth, exhibits extreme salivation, or sometimes the mouth may be hot and dry. The appetite is lost and an offensive gas is belched forth. The animal loses control of the limbs, and sometimes the muscles of the neck are contracted on one side. It becomes stupid, falls, and rarely rises again. It may die in a few hours or lie for a week. He reports a post-mortem in which the intestines and surrounding fat were green immediately after death. The arteries and smaller vessels in the limbs were gorged with thick dark blood. The lining of the first stomach was worn and ulcerated in patches and in some cases decomposition seemed to have commenced; it was very soft and could be peeled off the muscular layer in big pieces. The lungs and heart were almost bloodless, and the brain was purplish, soft, and pulpy. He states, however, that the symptoms vary in different cases. He lists as loco plants *Oxytropis lamberti*, *Leucocrium montanum*, *Fritillaria pudica*, and *Zygadenus elegans*.

In 1889 Dr. Mary G. Day, in two articles in the New York Medical Journal, describes the process which she followed in attempting to separate a poison from loco plants and gives the result of some pharmacological experiments with a decoction. She fed the decoction to a cat and found disturbing symptoms in two days. There was less activity, the coat became rough, the appetite was lost, fondness for loco was induced, and there was diarrhea, retching, and vomiting. Emaciation increased until the eighteenth day, when convulsions

occurred. There were alternate periods of excitement and quiet for 36 hours, when the posterior extremities became paralyzed, and the animal died in about two hours. The post-mortem examination showed ulcers in the stomach and duodenum. The heart was in diastole and anemic. In the second case she found similar symptoms but no periods of excitement. This cat died on the thirteenth day. Two cats were confined and treated in the same way, except that one was fed loco. The loco-fed animal acquired the disease, while the other remained well. In Michigan experiments on frogs and kittens caused nervous twitchings and death. A jack rabbit was fed on milk and grass, then fresh loco was substituted for the grass. It acquired the habit and died in ten days, with the head thrown back and with the stomach ruptured. Doctor Day states that more poison is present in the plants in the fall and winter. She reached the following conclusions:

1. There is some poison in loco weed which may cause the illness, and, if sufficient quantity is taken, the death of an animal.
2. This poison is contained in the decoction obtained from the plants, and by systematically feeding it to healthy cats cases of loco disease may be produced.
3. A taste for the green loco weed may be experimentally produced in the jack rabbit.
4. From the large quantity of the plant or of the decoction required to produce the disease, the poison must be weak, or, if strong, it must be in very small amount.

In the Druggists' Bulletin of 1889 is a résumé of the work on loco up to that date. Miss Day's work is criticized as valueless because of the lack of control experiments. The article is generally skeptical in regard to the existence of a specific poison in the loco plant.

In 1889 Power reported upon a chemical examination of loco, and later, in 1891, Power and Cambier made another report. The general result of their work was negative. They showed both in *Astragalus* and *Crotalaria* a very small amount of toxic alkaloids.

In 1889, in the Druggists' Bulletin, Professor Sayre reviews his preceding work in regard to the loco plants, gives drawings of two species, *Oxytropis lamberti* and *Astragalus mollissimus*, and reports the detail of a chemical examination of these plants. He also states that he has performed certain physiological experiments, but does not report them in this paper. He gives quite fully the result of the work of Doctor Ott, and then states that his own physiological experiments have all given negative results. He does not claim that the weed is not poisonous, but apparently does make the claim that nothing is proven as yet in regard to its poisonous properties.

Curtice, in 1889, in his report upon the animal parasites of sheep, quotes the report of the autopsy by Faville on locoed sheep, in which he found numbers of *tæniæ*. The author says that the description of locoed sheep applies equally well to those infested with *tæniæ*; that it is difficult to separate the symptoms of the two diseases, and

many cases of locoed animals are victims of tapeworms. That the *tæniæ* may tend to produce depraved appetites or the morbid craze for particular food is also a reason for suspecting that the loco disease may depend in part on the tapeworm disease.

Sayre, in 1890, in the report of the Kansas board of agriculture, gives the result of Miss Day's paper, in which she tells of the separation of crystals which she considered the poisonous principle. Sayre produced the same crystals and considers them inorganic. He says he himself has separated a crystalline organic substance but has not carried the investigation further.

An article by Pammel, in 1891, states that *Astragalus mollissimus* and *Oxytropis lamberti* are called loco plants by ranchmen. *Crotalaria* is the cause of loco in Iowa. In Australia a species of *Gompholobium* is a loco. He adds, also, as loco plants *Malvastrum coccineum* and *Physostigma venenosum*. He does not distinguish any difference in the specific effects of *Astragalus* and *Aragallus*. He also calls *Stipa viridula* a loco weed.

In 1892 McCullaugh gives a description of the pathological effects in locoed horses. He says that all herbivorous animals, even antelopes, are liable to the disease. The disease was first noticed west of the Rio Grande years ago. Since then the weed has been carried by birds and the continuity of the wind to the upper Missouri, into old Mexico, the Gulf of California, and California. The action of the plant is similar to that of alcohol or opium. The weed becomes green while the snow is on the ground. Among the first symptoms are loss of flesh, general lassitude, and impaired vision. The last may be taken as the first symptom. After the animal begins to become viciously shy an exostosis appears in the middle and superior portion of the frontal bone, becoming at times as large as a pullet's egg, and remains permanently. The animal exists in this condition from ten to eighteen months, but sometimes it will live not over three months. As the disease advances the animal loses muscular power and tetanic symptoms appear. It will invariably fall if suddenly startled. The muscles of the mouth and tongue become partially paralyzed, so that it is impossible to take food. The author has many times offered water to locoed horses that he knew had not drunk in five or six days. They would sometimes gaze stupidly for hours at a time at a trough of water, but make no attempt to drink. The true cause of death is starvation.

In 1892 Sayre, in the Thirteenth Report of the Kansas board of agriculture, after a résumé of his previous papers, states that he has been able to separate an alkaloid, but that it was not poisonous, and his conclusions are that there is a disease having a mysterious connection with *Astragalus* and allied genera. He quotes a letter from Professor Harshbarger, of Topeka, which states that animals hunt

loco, become nervous and later crazy; that they bite at water instead of drinking it, their eyesight becomes affected, and they become vicious and emaciated. Sayre then gives Harshbarger's opinion in regard to loco, which is skeptical and negative. He suggests that the disease may be caused by other things, as hot weather or stagnant water.

He also quotes a letter from Mr. Mayrath, of Dodge City, who states that he had 18 cattle eating loco in November, and that all died before May 1, although well fed. He says that cattle and horses eat loco for the most part in the late fall and winter; that they eat it grubs, eggs, and all. He suggests a connection of the grubby stem with the disease, and says he has been told of a man who was killed inside of twenty-four hours by loco tea.

Sayre then gives a résumé of Doctor Mayo's paper, and in conclusion gives an instance of a horse having loco disease, although it had never seen the loco plant.

Schuchardt, in 1892, in the *Deutsche Zeitschrift für Tiermedizin*, has an article on the loco disease of horses and cattle, in which he gives a careful and accurate résumé of the literature up to date, but adds nothing to what has been said in earlier publications.

In the same year Dr. B. F. Stalker, in the *Medical Current*, Chicago, states that as early as 1849 the Indians along the Missouri River told of a plant found in Kansas, Texas, and along the foothills as far north as Wyoming which would produce death, preceded by erratic forms of excitement. They said it affected cattle and horses, and the Mexicans claimed a similar effect on man. The author describes *Astragalus mollissimus* and *Oxytropis lamberti*, and states that abortion is sometimes produced by eating loco.

O'Brine, in 1893, in a bulletin of the Colorado State Agricultural College, gives a review of preceding literature, quoting especially from Faville. In 1889 *Astragalus mollissimus* and *Oxytropis lamberti* were fed to a sheep at the college, with no results. He states that the Colorado law offering a bounty for the destruction of loco plants was passed March 14, 1881, and repealed in April, 1885. It cost the State \$50,000 a year.

O'Brine tried to separate the poisonous principle, and failed. He found the same reactions for alfalfa that he obtained for loco. He made tests with rabbits, with no results. He made a number of autopsies near Livermore, and found a clot at the base of the brain in all cases. He suggests that the effects of the loco are not direct, but dependent upon some digestive processes.

Williams, in 1893, in the bulletin of the South Dakota Agricultural College, gives a list of the plants supposed to cause loco in South Dakota. They include *Astragalus mollissimus*, *A. lotiflorus*, *A. bisulcatus*, *A. haydeniensis*, and *Oxytropis lamberti*. He describes the plants and general effects on the stock, quoting Vasey and Mayo.

In 1893 Professor Mayo published a general article on the subject of loco, in which he not only described the symptoms in much the same terms as former authors, but gave the results of some personal examinations of his own. He says that the old inhabitants of south-western Kansas say it is more abundant upon ranches where Mexican sheep were pastured in an early day. He found on the roots two species of Tineidæ, and upon the plant a Bruchus and a Curculionid. He tried extracts of the dried plant and the fresh leaves on guinea pigs, but with no results. Both guinea pigs and cattle refused to eat the plant. He ate it himself with no ill effects, and concluded that the weed was not the cause of the disease, but when he made observations in the field he changed his mind. He found that animals acquire a taste, wander off alone, lose flesh, and have a stiff and stilted action of the legs. When lying down, they have difficulty in rising, the head trembles, the dependent parts of the body swell; they have a vacant stare, though they are not blind, and they do not shed their hair in the spring. They can be led only with great difficulty, shy violently, and are subject to crazy fits, especially when driven hard. Locoed animals never fully recover.

In post-mortems he found a large amount of serum in the body cavity, the intestines atonic, spleen small, liver small, pale, and adherent to the diaphragm. The rumen was full of loco. The meninges of the brain were thickened and congested. The brain was smaller than normal, and firmer, and the gray tissue was darker than normal and thinner on the cerebrum. In the central white substance of one brain were three small gelatinous translucent spots about the size of a small pea. Sections of the brain showed atrophy. Purkinje's kidney cells in some cases had disappeared; in other cases the processes were atrophied. There were no changes in the spinal cord. His conclusion was that there was no evidence of a narcotic principle in the plant, and that the animals died from malnutrition or mal-assimilation produced by eating the loco plants. The cause of the delirium may be clots or thrombi in the brain, as these are apt to be formed during wasting and debilitating diseases. He suggests as treatment removing the animals from loco plants and giving them some good condition powders.

In the Medical Century of 1893 Doctor Givens writes on the subject of loco, or "crazy weed," or *Astragalus mollissimus*. He describes the plant in general and the effect upon animals of eating, much as had already been described by preceding authors. He then states that he had secured from Colorado some of the dried root of *Astragalus mollissimus*, from which he prepared a tincture and tested its effect when given as a medicine upon three cases of insanity, each of which had hallucinations of sight and hearing. The net result of his investigations was that loco has no effect on insane patients, and

there is no reason to think that it can be used to advantage in the treatment of mental disorders. He concludes that it is harmless, even when taken in large doses, although it may at the same time have a poisonous action on herbivorous animals, as claimed by others.

In the report of the Canadian Experimental Farms in 1893 Fletcher mentions the fact that the subject of loco weeds has been brought to their attention by the poisoning of sheep and lambs in Manitoba, and suggests that owners of herds should send in specimens of the plants in order that the reports may be verified.

W. Thornton Parker, in 1894, describes the symptoms of loco poisoning as seen by him in Texas and New Mexico. Describing the plant, which seems to have been *Astragalus mollissimus*, he suggests that beneficial results can be obtained by treating the affected animals with muriate tincture of iron with accessories of hygienic treatment.

In the same year Professor Mayo describes much more fully than in his earlier article, and very accurately, the symptoms of loco poisoning. He speaks of the dropsical swellings which sometimes occur in the dependent parts of the body. Locoed animals walk with a peculiar creeping gait that is recognized by those familiar with the disease. No poisonous principle was found, and he reiterates his former conclusion that the loco disease is the result of malnutrition.

In 1895 Ruedi reports an examination of supposed locoed sheep in Estes Park, Colo. He found their temperatures low—between 86° and 100° F.—with breathing stertorous, pulse slow, abdomen distended, and diarrhea present. In the autopsies he found them anemic, the intestines filled with gases, the liver enlarged and filled with blood, the kidneys congested, and the bladder distended. The heart was in diastole and the brain bloodless. He prepared solutions and fed rabbits. All passed through a period of excitement which the author explains as caused by the brain anemia, followed by a period of dullness. They were observed for a period of ten days. None died, but they were killed for autopsy. In all cases there was congestion of the abdomen and anemia of the brain. He separated a base which he called "locoïn" and an acid, and states that locoïn is not poisonous, but that the acid is. He refers to the loco as "white loco" or *Astragalus mollissimus*. It is difficult to tell what species he was dealing with, but it seems more likely that it was *Aragallus lamberti*. It may be said in regard to his work that there seems to be no evidence that the sheep examined by him had eaten loco, and the symptoms and the autopsies lead the present author to conclude that they did not have the loco disease at all.

In 1896 Nocholds, in the American Veterinary Review, gives his experience while driving a bunch of horses from southern to north-

western Texas. Some of them gradually acquired the loco habit. One of them wandered from the bunch, walked high, had a staring coat, glassy eyes, and could not be driven. Some of them showed the effect only when excited or driven hard. All were stupid. All affected animals were mares over 6 years old, and 23 out of 300 died within a year. The author thinks there is no cure.

In 1897 Mr. J. H. Maiden, in the Report of the Department of Agriculture of Sydney, New South Wales, in a discussion of poisonous plants in that locality, mentions the "indigo disease" of Australia as probably identical with the loco disease of the United States, and in connection with this he quotes O'Brine in regard to the loco disease.

In 1898 Professor Sayre published in the Kansas Medical Journal a repetition of his former statements, and concludes with this statement: "We feel warranted in saying that the so-called poison is a development within the animal, and not a product preexisting in the weed itself."

In 1899 Prof. S. B. Nelson, of the Washington Agricultural College, in a report on experiments on feeding wild plants to sheep, states that he has fed small amounts of *Astragalus spaldingii*, *Astragalus palouensis*, and *Astragalus dorycnoides*, but without any bad results.

In the Annual Report of the Department of Agriculture of the Northwest Territories in 1899, T. N. Willing refers to the abundance of *Oxytropis lamberti* in the Territories and expresses his surprise that there is not more trouble from it.

In an article on the ethno-botany of the Coahuila Indians of southern California, Barrows in 1900 refers to the genus *Astragalus*, known as "rattlesnake weed" or "loco weed" in California. He says that several species are poisonous to cattle, sheep, men, and horses. One species, he states, is used by the Coahuila Indians as a flavoring principle, and according to another author dry pods are pounded up and mixed with beans and perhaps other articles of food as a spice.

In Law's work on veterinary medicine, published in 1901, is a general review of the subject of loco, concluding with a special report of Miss Day's work.

In the Annual Report of the Bureau of Animal Industry for 1900 E. V. Wilcox states that *Astragalus spicatus* is the most important loco plant of Montana. Besides this species there are *Astragalus splendens* and *Astragalus lagopus*, which are harmful. He disproves the belief that the loco disease may be caused by alkali, and gives an account of the characteristics of the disease and the details of an acute case of a sheep which died in four days. He states that the disease in cattle is rare. In autopsies on sheep a slight congestion of the brain is noticed, the fatty tissue is reduced, and the muscles

pale. Locoed sheep may be fed and fattened for mutton, but never fully recover from the disease.

In the report of Chesnut and Wilcox on stock-poisoning plants of Montana, in 1901, is a discussion much like that in the preceding paragraph. They state that the most reliable observations on the subject of loco disease indicate that it is the result of eating undue quantities of certain weeds, and express the belief that the plants contain a poisonous principle which is harmful to domestic animals. They report two experiments, feeding a Belgian hare and a sheep with extract of loco, but without results except slight narcotic effects in the rabbit.

In 1901 Mr. Maiden repeats the statements made in 1897 in the Report of the Department of Agriculture of Sydney, and adds that in all probability the "nenta disease" of South Africa is identical with the "pea-eating disease" of Australia and the loco disease of the United States.

In the report of the Oklahoma Agricultural Experiment Station of 1902 are some brief statements in regard to loco. Loco is distributed over the western half of the territory and causes considerable loss.

In the Proceedings of the Pacific Northwest Wool Growers' Association of 1902, Professor Blankinship tells briefly about the effects of eating *Oxytropis lamberti* and gives its distribution in Montana. He states particularly that the loco habit is taught to one animal by another. Hence, he says, it is very important that animals that have commenced to eat the weed should be separated from the rest of the herd.

T. N. Willing, in the Annual Report of the Department of Agriculture of the Northwest Territories in 1902, gives a cut of *Astragalus lamberti* and says that *A. lamberti* and *A. splendens* are abundant from Manitoba to the Rockies. He gives a short description of the symptoms of loco poisoning. Cattle are seldom affected.

In 1903 Professor Blankinship, in a bulletin of the Montana Agricultural Experiment Station on loco and some other poisonous plants, treats rather fully of loco in Montana. He states that the loco plant in Montana is the *Oxytropis lamberti* Pursh., or *Astragalus spicatus* (Hook.) Rydb. After referring to the distribution of the plant generally, he says that in Montana it is found up to an altitude of 8,000 feet in the country east of the Continental Divide, between Livingston on the west and Billings on the east, the mountains on the south, and north to the vicinity of the Little Belt and Highwood Mountains. Sheep are the principal sufferers from loco in Montana, horses and cattle being rarely affected, and it is the young sheep and colts that are affected rather than the older ones.

He then describes the symptoms of the disease, and, taking up the subject of prevention, reasons that because it is the younger animals that are principally affected, especial care should be taken of them. In regard to the extermination of the plant, he says it is possible to accomplish this by digging it out, as has been proved in one or two specific cases. This should be done when the plant is in bloom in order to kill the seed crop.

In 1903, Van Es and Waldron, of the North Dakota Experiment Station, in an article entitled "Some Stock Poisoning Plants of North Dakota," give a general description of the symptoms of loco poisoning which adds nothing to that of previous publications. They state that the loco plants are *Aragallus lamberti* and *Aragallus splendens*, the former being generally distributed in the State, while the latter occurs on more sterile soil and is reported only from a few counties of the State. Neither plant has caused any large amount of damage in North Dakota.

In 1904 Sayre published two papers. In the first, entitled "What is Insanity in Lower Animals?" he suggests that the symptoms of loco disease may be due to anemia, cerebral congestion, inflammation of the brain or its membranes or of the membranes enveloping the spinal cord, or to forage poisoning. In his second paper, published in the Journal of the Kansas Medical Society, he gives an analysis to determine the food value of the loco plant, but does not discuss the results of the analysis. In this paper he broaches as theories to explain the effect of loco, (1) irritation by hairs; (2) disturbance to digestion, such as might happen from any food in a weakened condition of the animals in spring; and (3) a poison developed in the process of digestion.

In 1904 J. E. Payne, of the Agricultural Experiment Station of Colorado, in a report on cattle raising on the plains, states that there is no evidence that cattle eat largely of the loco weed or are affected by it. Where these plants are prevalent the range is very poor. He further says that the talk about loco is kept up by cattle owners to keep out settlers. A number of unnamed diseases may be included under the term "loco."

In 1904 Doctor Marshall published in the Johns Hopkins Hospital Bulletin a résumé of the results of his work of the preceding two years, while in the service of the United States Department of Agriculture, in feeding locoed sheep in Montana. His conclusions may be summarized as follows: The loco disease affects horses and sheep, and occasionally cattle and goats. *Astragalus mollissimus* and *Aragallus spicatus* are the commonest loco weeds. Ranchers consider as important accessory factors: (1) Age; the disease appears before the end of the second year; (2) insufficient feed; (3) insufficient supply

of water; lack of salt; (4) general conditions of health; healthy animals never acquire the disease. He then describes the symptoms.

In this investigation only sheep were examined; all were lambs or yearlings. They did not prefer the loco. Many had bronchitis, conjunctivitis, etc. Usually the fleece was rough. All were infected with parasites. The parasites found were *Thysanosoma actinioides*, *Haemonchus* sp. (in the fourth stomach), *Metastrongylus filaria* (in the bronchioles), *Sarcocystis tenella* (in the muscles), and *Cysticercus tenuicollis* (in the peritoneal cavity). The fringed tapeworm was so abundant as to dilate the bile ducts.

He concludes that there is no true loco disease of sheep produced by the weed, but that the so-called locoed animals suffer from bad feeding, insufficient care, and a variety of other diseases, the most important of which are the parasitic diseases.

Fletcher in 1905, in evidence given before the committee on agriculture and colonization at Ottawa, Canada, stated that the loco weeds were abundant in Alberta and through to Manitoba, but are not as injurious as in Montana. He has never seen a case of loco disease in Canadian-bred animals.

In 1905 Professor Linfield, director of the Agricultural Experiment Station of Montana, in a report of a series of experiments on sheep feeding, compares the effect of feeding upon a flock of locoed sheep with the effect produced upon a flock of normal sheep. He states as his general results that the locoed sheep gained about as much as the normal sheep, but inasmuch as they were very thin at the start, it took about twice as long to get them ready for market. All these locoed sheep were treated with a vermifuge at the beginning of the experiment, and the author implies that their locoed condition may have been the combined result of poor feed and parasites. His work may be criticized, inasmuch as his figures as to gain in weight are based on the surviving locoed animals. He started with 42, and only 29 remained at the end of the experiment. He states that nearly half of this lot was lost through natural death or by killing for post-mortem examinations. The fact, however, remains that his figures are based on the best of the flock, and the statistical results of the feeding experiment would probably not have appeared as favorable for the locoed animals if the whole number had been considered.

Again, in 1905, in the Proceedings of the Kansas Academy of Sciences, Sayre published a partial bibliography of the subject and repeated the report of the analysis and his theory of the causation of the disease by irritation by the hairs of the plant.

Prof. L. G. Carpenter, director of the Agricultural Experiment Station of Colorado, in the eighteenth annual report of the station (for 1904-5) states that considerable work has been done by Payne

on the subject of loco, which has made clear the necessary conditions of the experiment. He also refers to the cooperative work undertaken with the United States Department of Agriculture.

In this same publication Dr. George H. Glover, veterinarian of the Colorado Agricultural Experiment Station, gives a partial report upon the first year's cooperative work at Hugo.

Prof. S. B. Nelson in 1906 reports feeding small quantities of *Astragalus spaldingii*, *A. arrectus*, *A. purshii*, and *A. reventus*, but with no results. He suggests that longer feeding might have produced more positive results.

Prof. L. G. Carpenter, in the report of the Colorado Agricultural Experiment Station for 1906, published in 1907, gives a résumé of the work done by the United States Department of Agriculture at Hugo.

Doctor Glover, in a report of the veterinarian to the director of the Colorado Experiment Station, published in 1907, makes a brief statement also in regard to the work at Hugo.

SYMPTOMS MENTIONED IN LITERATURE.

In the preceding pages we find noted a very large number of symptoms of loco poisoning. The list below gives most of these, omitting those found by Doctor Gee in his experiment on his class, as they were almost infinite in number as well as contradictory. In this list the symptoms are arranged purposely in such a manner as to show their contradictory character.

Congestion of abdomen.	Fourth ventricle of brain with blood clot.
Abortion.	Breathing short.
Anemia.	Coat rough.
Loss of appetite.	Winter coat retained.
Arteries gorged with thick, dark blood.	Constipation.
Bile thin and watery.	Diarrhea.
Bladder with inner coat softened.	Convulsions.
Bladder distended.	Craziness.
Blindness.	Hallucinations.
Body cavities with an excess of serous fluid.	Lack of muscular coordination.
Brain atrophied.	Emaciation.
Brain bloodless.	Exostosis of frontal bone.
Brain with the membranes inflamed.	Eyes glassy.
Brain congested.	Eyes dull and staring.
Pia mater injected.	Pupils dilated.
Gray tissue of brain darker than normal.	Vision impaired.
Gray substance of brain red and edematous.	High temperature.
Brain tissues soft and pulpy.	Low temperature.
Sinuses of brain filled with fluid.	Frothing at the mouth.
Lateral ventricles of brain holding serous effusion.	Shaking head.
	Impaired hearing.
	Heart of increased size.
	Heart with inflamed valves.

Heart bloodless.	Rearing and plunging.
Heart with reduced action.	Shying.
High stepping.	Solitary habit.
Hyperæsthesia.	Spinal cord edematous.
Intestines filled with gases.	Spinal cord membranes inflamed and adherent.
Intestines congested.	Spleen small.
Intestines green.	Excitement followed by depression.
Intestines with tissues degenerated.	Stomach ruptured.
Kidneys congested.	Third stomach (psalterium) softened.
Purkinje's cells in kidneys lacking.	Second stomach (reticulum) softened.
Difficult to lead.	First stomach (rumen) lining worn, ulcerated, and decomposed.
Liver dense.	Swelling under the jaw.
Liver small.	Swelling of dependent parts of body.
Liver enlarged.	Teeth loose.
Lungs bloodless.	Teeth not loose.
Narcotized.	Tremors.
Stiff neck.	Tendency to turn either to the right or to the left but not in both directions.
Irritability of motor nerves.	Urine offensive.
Decrease of irritability in sensory nerves.	Vomiting.
General derangement of nervous system.	Weakness across loins.
Paralysis of mouth or tongue.	Weakness and staggering gait.
Paralysis of hind legs.	
Omentum inflamed, with tumors.	

While this list is confusing because of the number of items and the contradictions involved, yet in regard to some of the symptoms there is great uniformity. The following symptoms have been noted by a large number of observers, and in regard to them there is a fairly general agreement:

Slow, staggering gait.	Lack of muscular coordination.
Rough coat.	Extreme nervousness, shown in shying, rearing, etc.
Staring look.	
Emaciation.	

REMEDIES PROPOSED.

The remedies suggested for the loco disease by different writers, as would be expected from the indefinite knowledge of the subject, have been of various kinds, many of them having no logical basis. Among them are the following:

1. Potassic bromid, belladonna, and bleeding.
2. Amputation of the tail, which, of course, is simply one form of bleeding.
3. Potassium iodid and quinin, with strychnin; in some cases it may be desirable to add jaborandi or pilocarpin.
4. Blister on the top of the head with the administration of 60 grains of calomel every second or third night. The calomel should be followed by sulphate of iron.
5. A prescription of belladonna, calomel, licorice, and glycerin.
6. A prescription of iron sulphate, gentian, muriate of ammonia, and potassic nitrate.

7. In acute cases, permanganate of potash, sulphate of ammonia, morphine as an antispasmodic. In chronic cases, remedies to affect the stomach, as common salt, hydrochloric acid, and gentian root.

8. Muriate tincture of iron with hygienic accessories.

CONCLUSIONS FROM LITERATURE.

From the foregoing review of the literature we may deduce the following conclusions:

1. There is a disease common among horses, cattle, and sheep in the semiarid region of the West, the animals affected showing these characteristics: Slow, staggering gait, rough coat, staring, vacant look, and emaciation. They have hallucinations, can not be led or backed, show more or less lack of muscular coordination, gradually lose flesh, and die. They show a hypersensitive condition of the nerves, so that if a horse affected with loco disease is suddenly startled he may rear and fall over backward. Cattle show less pronounced symptoms, but in general are affected like the horses. The characteristic of a locoed steer is the shaking head, together with the other nervous phenomena. To these symptoms may be added some others upon which there is no general agreement.

2. The cause of this disease is quite uniformly ascribed by the stockmen to plants known by them as loco plants, of which the principal ones are *Astragalus mollissimus* and *Aragallus lamberti*. Similar properties are ascribed to other closely allied species in Montana, Wyoming, Arizona, and California.

3. Scientific investigations of the subject have led to very contradictory results.

It will be noticed from the review of the literature that in the chemical and pharmacological examination Prescott got no positive results. The same is true of the experiments made by Gee. Kennedy found no poisonous principle and fed the loco to a dog with no results. Day got from the extract fed to cats definite loco symptoms, and similar results from her experiment with a jack rabbit. The work of Power and Cambier led to only negative results. Sayre made numerous experiments on himself and animals, and has reached the general conclusion that the weed possesses no poison. It will be seen that the only positive proof of the poisonous character of the plant was obtained by Doctor Day. The preponderance of laboratory tests made up to the time that the Bureau of Plant Industry undertook its investigations was, therefore, against the poisonous character of the plant.

The results of the study of locoed cattle, horses, and sheep in the field are no more conclusive. The reports of post-mortems show few lesions common to all cases, or even to any considerable number of them. There is a fairly general agreement as to some abnormal

condition of the brain and the nervous system, but so far as the other organs of the body are concerned, the reports do not agree with each other. There seems to be a general feeling of skepticism among those who have seen cases in the field as to the actual poisonous properties of the plants. The following have been suggested as the possible causes of the disease:

1. Starvation.
2. Irritation of the stomach by the hairs of the leaves.
3. Penetration of the stomach by the sharp hooks of the seed pods.
4. The loco absorbs the juices of the stomach, collects as a dry mass, causes pressure upon the circulation, and finally death.
5. The formation of balls in the stomach from sand taken in when the grass is short (bezoars), and the consequent irritation and inflammation due to the presence of this foreign substance.
6. Impaction of the intestines by twigs, etc.
7. Fermentation of the materials in the stomach.
8. Tapeworms.
9. Other parasitic worms.
10. Eggs or bots.
11. Trypanosomes.
12. Some poisonous principle in the plants.

To these possible causes might be added some suggestions made by stockmen which have little foundation. It is generally known that the loco plants are attacked by a considerable number of insect enemies, of which more will be said later. Among them are certain insects whose larvæ live in the roots and stems of the plants and have been noticed for a great many years by the stockmen. Many of the stockmen consider that these "worms," as they call them, are connected directly or indirectly with the loco disease. Some of them state that the eggs of the worms are taken into the body of the animal, hatch out there, and live in the alimentary canal; evidently confounding the ordinary parasitic worms of animals with these insect larvæ. C. D. Steele, in *Farm and Ranch*, in 1901, suggests that these "worms" consume the food in the stomach and thus produce starvation. One correspondent of our office has elaborated an interesting theory to this effect: That the cattle and horses eat the loco plants upon which are the worms, as he calls them, and the eggs of the worms. These eggs hatch out in the stomach of the locoed animal and crave loco for their own food, and it is to supply food to satisfy this craving of the worms that the horse or the steer frantically seeks loco.

It will be remembered that Sayre in his 1904 paper broaches three distinct theories: (1) Irritation by hairs; (2) disturbance of digestion, such as might have been caused by any food in the weakened condition of the animals in the spring; and (3) a poison developed in the process of digestion.

Marshall's investigations led him to conclude that the loco plant was not the direct cause of the disease and that many cases of so-

called loco disease were really caused by animal parasites. This seemed particularly probable in the case of sheep, which all through our western region are infested with *Æstrus ovis* and with *Thysanotoma actinioides*, as well as certain other animal parasites.

It will be seen that the loco problem up to the time when the review of this literature closed was an open one, with the presumption in favor of the nonpoisonous character of the plant, and in taking up the investigation of this subject it seemed necessary as the first step to determine positively whether the loco plants were really the cause of the so-called loco disease.

PLANTS KNOWN AS LOCO PLANTS.

A large number of plants have been known as loco plants. The following list is taken from the literature on the subject. It is to be presumed that there have been some errors in determination, but the length of the list shows that loco is a term which has been applied quite widely. By general agreement, however, the term, outside of California, New Mexico, and Arizona, is now rather generally confined to the two species, *Aragallus lamberti* and *Astragalus mollissimus*. In some localities the term "loco" is confined to *Astragalus mollissimus*, but this is generally in places where *Aragallus lamberti* is comparatively rare.

LIST OF SPECIES.

<i>Amarantus albus.</i>	<i>Crotalaria.</i>
<i>Astragalus bigelovii</i> Gray.	<i>Cystium diphysum</i> (A. Gray) Rydb.
<i>Astragalus crotalaria.</i>	<i>Datura stramonium</i> L.
(= <i>A. oocarpus</i> Gray.)	<i>Diholcos bisulcatus</i> (Hook.) Rydb.
<i>Astragalus dorycnoides.</i>	<i>Diholcos haydenianus</i> (A. Gray) Rydb.
(= <i>A. succumbens</i> Dougl.)	<i>Fritillaria pudica</i> Spreng.
<i>Astragalus hornii</i> , A. Gray.	<i>Lathyrus polymorphus</i> Nutt.
<i>Astragalus lotiflorus</i> Hook.	(= <i>L. incanus</i> .)
<i>Astragalus lentiginosus</i> Dougl.	<i>Leucocrinum montanum</i> Nutt.
<i>Astragalus menziesii</i> Gray.	<i>Malvastrum coccineum</i> (Pursh.) Gray.
<i>Astragalus mollissimus</i> Torr.	<i>Orybaphrus.</i>
<i>Astragalus mortoni</i> Nutt.	<i>Oxytropis lagopus</i> Nutt.
<i>Astragalus palousensis</i> Piper.	(= <i>Aragallus lagopus</i> Nutt.)
<i>Astragalus spaldingii</i> Gray.	<i>Physostigma venosum.</i>
<i>Astragalus tridactylus</i> A. Gray.	<i>Psoralea cuspidata</i> Pursh.
<i>Aragallus lamberti</i> (Pursh.) Greene.	<i>Rhamnus lanceolata</i> Pursh.
<i>Aragallus sericeus</i> (Nutt.) Greene.	<i>Sophora sericea</i> Nutt.
<i>Aragallus spicatus</i> (Hook.) Rydb.	<i>Stipa viridula</i> Triv.
<i>Argemone mexicana</i> L.	<i>Zygadenus elegans</i> Pursh.
<i>Cannabis sativa</i> L.	(= <i>Anticlea elegans</i> (Pursh.) Rydb.

DESCRIPTION OF ARAGALLUS LAMBERTI.

Aragallus lamberti (Pl. I) is a perennial growing from a root which may extend 2 or 3 feet or more into the ground. It is acaulescent and the leaves rise to a height of a foot or more. The general habit of the plant is of erectness. The leaflets are oblong, lanceolate, or linear, and are covered with a variable amount of silky hairs. In some varieties this pubescence is quite dense. The flower scapes are longer than the leaves and bear spikes which are either densely or sparsely flowered. The flowers are large and variable in color; the more common color of the plants on the plains is white, but they may be distinctly purple. The variations are from white, through a light purple and lilac, to a distinct purple. In some cases the corolla

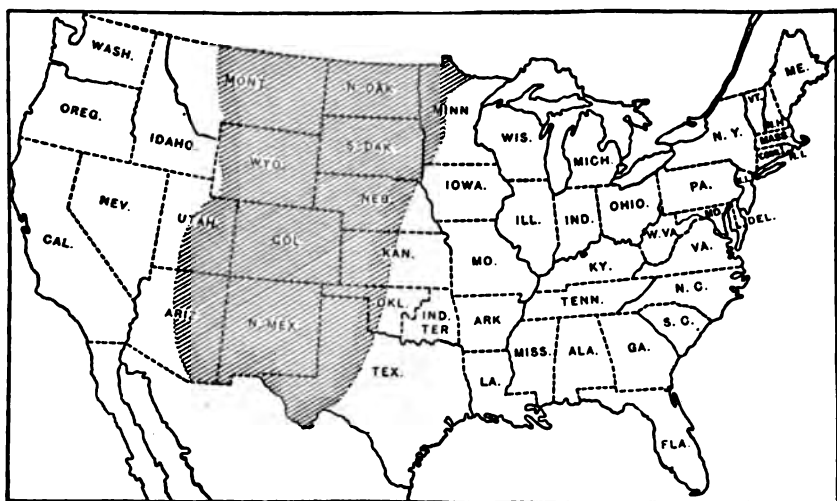


FIG. 1.—Distribution of *Aragallus lamberti* in the United States.

is white and the calyx red. In the mountain varieties there is the same variability in color, but the darker ones are more common, the predominating colors being deep violets and purples. The white varieties are commonly found in woods and near streams.

The plants are strikingly beautiful, and grow so abundantly that when in blossom on the plains large areas are as white as a field covered with snow, while in the mountains, although less abundant, the patches of several acres of bright purple and violet flowers are extremely attractive. The pods of the *Aragallus lamberti* are rather long, pubescent, and indistinctly two-celled. It grows most abundantly on the low hills of the plains, and in the pastures at the base of the mountains up to a height of perhaps 9,000 feet. It prefers a sandy soil.

Aragallus lamberti has an extremely wide range, as may be seen from figure 1. It is found from Alaska on the north down through

the Dakotas and as far east as central Minnesota; in Montana, Wyoming, western Nebraska and Kansas, eastern Colorado, Oklahoma, western Texas, New Mexico, and Arizona. In all these localities more or less damage has been reported, but it is in Wyoming, Colorado, and Arizona that the most trouble has occurred. It is abundant in the eastern foothills in Colorado, and on many of the mountain ranges horses are no longer turned out. It is especially abundant in parts of Elbert, Lincoln, and Cheyenne counties in eastern Colorado. In Arizona there is a large amount of this plant about the base of the San Francisco Mountains. It grows more abundantly east of the Continental Divide, but is by no means confined to this region. Considerable patches of it are found on the western slope in Colorado. In this region it has so far done very little damage, and most of the stockmen do not know that it occurs in that region at all.

The plant belongs to the semiarid region. It is not, however, entirely confined to dry soils and sterile regions. In the Black Hills of South Dakota it occurs in the forests and in soil which, during a part of the year at least, is full of moisture. The pubescence is much more marked in the dry regions. *Aragallus lamberti* is popularly known as "rattleweed" or "white loco."

The species *spicatus* (Hook) Rydb., *albiflorus* Nelson, and *sericeus* Gray are considered by Prof. C. F. Wheeler, who has determined our plants, as identical with *lamberti*.

DESCRIPTION OF ASTRAGALUS MOLLISSIMUS.

Astragalus mollissimus (Pl. II) is a perennial growing from a long root like that of *Aragallus lamberti*. Unlike the latter, it has distinct stems in the older plants, although the young plants appear acaulescent. The plant is inclined to be decumbent, although young plants are sometimes quite erect. In this habit it differs markedly from *Aragallus lamberti*. Most of the plants are comparatively small, living apparently not more than two or three years. Under favorable circumstances, however, they may grow into large bushy structures, perhaps a foot high and from 1 to 2 feet in diameter. The leaflets are broadly ovate or elliptical, and densely pubescent. This pubescence is very much greater than in *Aragallus lamberti*. The flower scapes do not ordinarily much exceed in length the leaves, and bear rather dense spikes of deep violet or purple flowers. The flowers are much smaller than those of *Aragallus lamberti* and less showy. The pods are thicker than those of *Aragallus lamberti*, smooth, and distinctly two-celled.

Plate II, figure 1, shows the appearance of the younger plants and the characteristic appearance of the leaves, flowers, and fruit, while figure 2 of that plate shows the general appearance of the older



Fig. 1.—Plant in flower.

WHITE LOCO, OR RATTLE WEED (*ARAGALLUS LAMBERTI*).



Fig. 2.—Plant in fruit.

U.S. M.





Fig. 1.—Flower and fruit.



Fig. 2.—Habit of plant.

PURPLE OR WOOLLY LOCO (ASTRAGALUS MOLLISSIMUS)

Mr. U

plants, in which the decumbent character is more pronounced. It does not grow in as great abundance as the rattleweed, although occasionally a few acres may be pretty well covered. It grows best on an adobe soil, and where the two species—*Aragallus lamberti* and *Astragalus mollissimus*—grow near together, *Astragalus mollissimus* occupies the depressions and *Aragallus lamberti* is found on the hills.

Its range (see fig. 2) is not so wide as that of *Aragallus lamberti*. It occurs in Wyoming, Nebraska, Kansas, Colorado, Oklahoma, Texas, New Mexico, and Arizona. In the eastern part of the plains region of Colorado and in western Kansas and Nebraska *Astragalus mollissimus* is very abundant, while *Aragallus lamberti* is comparatively rare. The same is true of the Panhandle in Texas. In parts of New

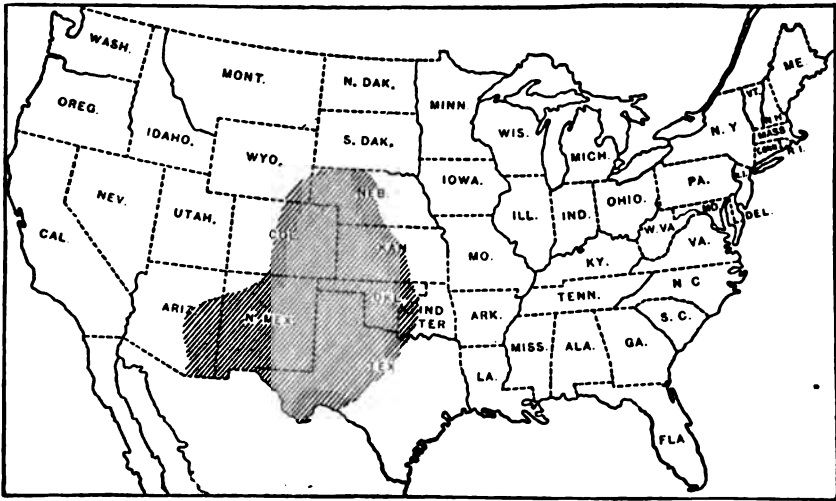


FIG. 2.—Distribution of *Astragalus mollissimus* in the United States.

Mexico it grows very luxuriantly. This is particularly true in some portions of the Estancia Valley and in the regions about Santa Rosa. It is found in less abundance in Arizona, and apparently in that Territory does very little harm. The most harmful of the loco plants in Arizona appear to be entirely distinct from the two species under discussion.

Astragalus mollissimus is popularly known as "purple loco," "woolly loco," or "Texas loco." Sometimes it is spoken of by the ranchmen as "the loco."

DISPERSAL OF ARAGALLUS LAMBERTI AND ASTRAGALUS MOLLISSIMUS.

When *Aragallus lamberti* ripens, the pods open while still on the flower scapes and the seed is scattered in the immediate neighborhood of the plant. The pods of *Astragalus mollissimus* are heavier

and may lie upon the ground, but because of their weight are not moved to any extent by the winds. Neither of these plants breaks off and becomes scattered in tumbleweed fashion. It follows that in the case of both of these species most of the new plants spring up in the immediate neighborhood of the parent plant, and the dispersal of the plants is a comparatively slow matter. It should not be assumed that the wind has no influence in scattering the seeds, for in a country of violent wind, seed, like everything else that is loose, will be carried about more or less. This is shown in the fact that *Astragalus mollissimus* is frequently abundant in and near wagon roads, doubtless because the moving seeds have been arrested in the depressions. Loco is said to be especially abundant near old sheep trails. This may be partly because of the seeds carried in the droppings of the sheep and partly because of the arrest of the moving seeds by the depressions and the planting of these seeds by the feet of the sheep.

It is said by some (Blankinship, 1903) that the bison were instrumental in the distribution of the plants either through their droppings or because of their habits of wallowing, by reason of which they may have carried the seed to great distances. These statements in regard to the distribution by sheep and bison, however, are entirely theoretical, for no exact observations have been made.

RELATIONS OF LOCO PLANTS TO FERTILE SOIL.

Incidentally it may be remarked that the loco weeds, like other leguminous plants, have root tubercles, and presumably are efficacious in adding nitrogen to the soil content. Mr. W. S. Pershing, of Limon, Colo., states that he has noticed that he has had especially good crops when he has used land on which loco has been growing. It seems very possible that while loco is a curse to the stockmen it may be of some benefit to the farmer.

EXPERIMENTAL WORK LIMITED TO TWO PLANTS.

The experimental work on which this paper was based was limited to the two species *Astragalus mollissimus* and *Aragallus lamberti*. There is reason to believe, however, that a considerable number of related plants may possess the same properties.

Horses in Arizona and New Mexico die with the typical symptoms of loco poisoning, and post-mortem examinations show lesions similar to those found in the victims of *Astragalus mollissimus* or *Aragallus lamberti*, but the loco plants are different species from those found farther north. While there is little reason to doubt that these plants possess the same poisonous principle and produce the same result, experimental work has not been undertaken to demonstrate these facts.

Through many parts of the West horses are said to become "saged" and to have many of the symptoms of loco poisoning. This has been noted by Mayo. The author has seen some cases of so-called saged horses, but there was no means of proving that they had not been eating loco plants. The testimony of stockmen who have what seems to them clear evidence of the production of these symptoms by the sages is not to be lightly set aside. It is hoped that it may be possible to do some convincing experimental work on these plants.

Reference should be made, in passing, to the poisonous effects of *Swainsona galegifolia*, known as the Darling pea or indigo plant, and other species of *Swainsona* which are said to produce in Australia symptoms like those caused by the American loco plants. MacOwan considers the "nenta" of the Cape as produced in a similar way.

PART II.—EXPERIMENTAL WORK.

THE PROBLEM TO BE SOLVED.

It will be seen from the foregoing that when, at the earnest and persistent solicitation of the stockmen, it was decided by the Department of Agriculture to make a thorough study of the subject, there was very little definite knowledge to start upon. In spite of the fact that the subject had been under investigation for a third of a century, there was no consensus of opinion even in regard to the poisonous or nonpoisonous character of the plants themselves. The stockmen with very few exceptions were convinced that the plants were the cause of the disease. On the other hand, laboratory investigations were contradictory and the investigations in the field were not conclusive. The problem, as presented to the Department then, involved:

1. The definite determination of the poisonous or nonpoisonous character of the loco plants. This included (a) field experiments in feeding animals under normal conditions, with proper controls, to find out whether the disease could be produced by the loco plants, and (b) a pharmacological examination of the plants to determine whether a poisonous principle could be extracted, and if it could be extracted to determine its properties. The pharmacological work was entirely in the hands of Dr. Albert C. Crawford, who has made an independent report of his investigations.^a

2. A careful examination of locoed animals, with numerous post-mortems to determine the pathological lesions, with the end in view of making an accurate and fairly complete diagnosis of the disease from the pathological side.

3. An attempt to find some remedy for the trouble. This branch of the subject presented two aspects: (a) The suggestion of measures to eradicate the plant or to reduce its numbers, if it was found to be the cause of the disease; (b) the suggestion of remedies which could be given to the animals either for a cure or for lessening the effects of the disease.

It was evident that the investigation would have to be equipped in a very broad way. While the study was primarily of the loco

^a Bulletin 129, Bureau of Plant Industry.

plants, it was necessary to be provided with means of making post-mortems, giving special attention to the presence of animal parasites; pathological tissues must also be collected and examined, the blood must be studied, and an equipment for the cultivation of pathogenic bacteria must be on hand.

PLAN OF WORK.

To carry out the first point in the investigation, it was determined to secure the use of land somewhere in the loco area, and fence in one piece covered with loco thickly enough so that the animals in the pasture would be compelled to eat loco, but with grass enough so that there would be no question of starvation. Another piece of ground, as nearly like the first as possible, but free from loco, was to be used as a control pasture. In addition, animals should be confined in the corrals and fed on cut loco, cut loco and hay, etc. It would be necessary also to confine certain groups of animals to definite loco species, and thus determine the differences, if any, in their poisonous properties. Incidentally, it was desired to travel through the loco country and collect facts in regard to the losses from loco, the loco conditions in different places, and the distribution of the supposed poisonous plants.

LOCATION OF EXPERIMENT.

As Colorado has perhaps suffered more losses from loco than any other region it was determined to locate the experiment in that State. A cooperative agreement was entered into with the Colorado Agricultural Experiment Station by which the station agreed to furnish, in aid of the work, the stock to be experimented upon and the services of a veterinarian for consultation when called upon. After some time spent by Doctor True and the author in examining different areas of the State where loco was doing most harm, it was decided to locate a temporary station at Hugo, in Lincoln County. The choice of this location was determined, in part, by the fact that no part of the West has suffered more from loco than this section of Colorado, and in part because of the active interest in the investigation manifested by the stockmen of that neighborhood.

In this connection the author makes grateful acknowledgment of the courtesies extended and valuable assistance rendered by many persons, too numerous to mention by name, in the course of the investigations. The stockmen not only gave freely of their time in showing local conditions, but were always ready to furnish animals for autopsy and to assist in their examination. The county board of Lincoln County made liberal appropriations for the experiment.

Special acknowledgment, too, should be made of the assistance very freely given by the railroad officials in the territory visited; without this help it would have been impossible to handle the work with the funds at our disposal.

PRELIMINARY WORK.

During a preliminary trip through the State a considerable number of locoed animals were seen. The picture in the text of case 513 (fig. 3), a stunted colt seen near Wray, is typical of the locoed horses observed on this trip. In spite of many cases, where there was an abundance of feed, the conclusion was reached that in the great majority of cases starvation was the principal if not the only factor in the so-called loco disease. Prof. W. L. Carlyle, of the Agricultural Col-



FIG. 3. Case 513. A locoed colt, stunted in its growth by loco poisoning.

lege, accompanied us in our examination of the neighborhood of Hugo, and agreed with us that an abundance of good feed would greatly reduce the number of cases and perhaps eliminate the problem.

An old ranch house with its corrals was secured as laboratory headquarters for the work. A piece of ground of something over 200 acres, where

the *Aragallus lamberti* was especially thick, was prepared as a loco pasture and was so fenced that the animals should always have access to running water. Over the whole of this 200-acre pasture the *Aragallus lamberti* was distributed in very great abundance. It is doubtful if there was a square yard of the pasture which did not have one or more of the plants. It was also very nearly a field of *Aragallus lamberti* simply. There were in the depressions small patches of *Astragalus mollissimus*, but during the progress of the experiment the animals were watched carefully, and there is reason to think that none of them ate this plant in this pasture.

Another piece of ground near by was arranged for a control pasture. This piece of land had a comparatively small amount of loco upon it. A force was set at work to clear out this pasture so that it would be to all intents and purposes a loco-free piece of ground. During the summer men were sent over it two or three times in addi-

tion, in order to kill out the seedlings as they came up later in the season. Thus in this pasture we had a piece of ground very nearly like the loco pasture but without the loco plants. Inasmuch, however, as the loco-free pasture was somewhat lower, it is a fact that the grass was rather better than in the loco pasture. However, there was an abundance of grass in the loco pasture, so that we consider that the starvation element was entirely eliminated.

WORK OF THE FIRST SEASON.

Twelve steers and fifteen horses were received from the agricultural experiment station for experimental purposes. Part of these arrived at the end of April, 1905, and part the first week in May. By the end of the first week in May the experiment was fairly started. The animals were given individual brands and numbered, so that the notes of each animal could be kept under its own number. Five of the cattle, Nos. 1, 2, 4, 5, and 6, and four of the horses, Nos. 13, 16, 22, and 23, were put in the loco-free pasture and kept there through the season as controls. In selecting these controls the animals were taken by a chance selection in order that the loco animals should be just as good as the controls but no better. The controls had an uneventful history. All kept well and flourished throughout the summer, and came out in the fall in good condition.

Of the remaining animals part were put in the loco-free pasture to be drawn upon for corral experiments, and part were placed in the loco pasture. In order to induce the loco animals to eat a large amount of the weed, it was found expedient after the last of May to bring them into the corrals at night and keep them in until rather late the next morning. By this treatment they went to the pastures in the morning very hungry; and as hungry animals—and this is especially true of cattle—are apt to eat the most prominent plants, they would for an hour or two eat loco almost exclusively. After satisfying their first hunger they would commence to eat grass. It was found that they ate much less loco when kept in the pasture nights as well as days.

Astragalus mollissimus did not occur in sufficient amount near Hugo so that a pasture could be arranged where stock could be fed, and we found it necessary for experiments on this species to cut the loco and feed the animals in the corrals. A little later a considerable piece of ground was found on the Van Antwerp ranch, about 10 miles south of Hugo, where the *Astragalus mollissimus* was particularly luxuriant. This piece of ground was loaned for experimental purposes by Mr. Laurie, and was fenced in as a pasture. Two horses and two steers were placed in it on July 13. The pasture was visited every other day and the animals were watched to see what they were

eating. It was necessary to construct a lane from the pasture to a well so that they could get water, and in this lane there was a considerable amount of *Aragallus lamberti*. The animals ate this plant, but there is no evidence that they ate any of the *Astragalus mollissimus*. It was hoped that as the grass gradually became shorter they would be forced into eating the weed, but to our surprise within two or three weeks nearly all the *Astragalus mollissimus* was dead, having succumbed to insect enemies. On August 25 it was decided to take the animals out and abandon the pasture, as there was not enough of the loco left to form any prominent part of their food. There was no evidence that the animals in this pasture had eaten any of the loco even when it was most abundant, so the pasture experiment upon *Astragalus mollissimus* must be considered as a failure.

All the animals, when received in the spring, with the exception of one horse which died soon after its arrival in Hugo, were in apparently healthy condition, although rough and poor, as they had just come off the range and suffered more or less from short winter feed. As the result of good pasture, all commenced immediately to improve. The steers in the loco pasture ate *Aragallus lamberti* freely, and while we at first imagined that they were affected by it, it soon became evident that they were thriving. Their coats became smooth, they gained in flesh, and seemed to give evidence that the loco diet was an excellent one. The earlier autopsies, too, instead of showing the presence of intestinal parasites, as had been expected, apparently showed that loco animals had fewer internal parasites than would be expected in normal animals. Our skepticism in regard to the poisonous effect of loco was confirmed, and it appeared probable that we should prove that loco was a myth, or, at least, a misconception, and that the cause of the disease must be sought in some other direction. As the season progressed; however, occasion was found for a change of opinion, as will be seen after the description and detailed discussion of the cases.

The experiment was carried on through the season with no unexpected difficulties of a serious nature, with the exception of the appearance of glanders in the loco pasture. Suddenly one of the best of the horses developed this disease in an acute form and died after a short illness. This case was followed by another, which also died. Fortunately the disposition of the animals just at that time was such that there was reason to think that only one other horse had been exposed. This horse was isolated for a time, care was taken to give the pasture a chance for disinfection, and the experiment was resumed with no further untoward result. It is to be presumed that this case resulted from infection received before the animals arrived in Hugo.

A brief history is given in the succeeding pages of some of the more interesting cases. In these statements the results of the autopsies are not mentioned, as they are discussed in some detail later (see p. 95).

EXPERIMENTS WITH CATTLE.

Case 7.—This steer was pastured on *Aragallus lamberti* during the first part of the season. Later it was taken into the corral and fed exclusively on cut *Aragallus lamberti*. This was eaten very freely for a short time, when the animal showed distaste for it, and the experiment was tried of feeding *Astragalus mollissimus* in the corral. This the steer utterly refused to eat even when starved to it. When on occasional days the animal was turned out into the pasture it would eat grass and *Aragallus lamberti*. In order that it might not starve to death, it was afterwards turned into the *Aragallus lamberti* pasture, remaining in the pasture until October. In the middle of October it suddenly began to show weakness when walking, this being especially noticeable in its hind legs, and within two weeks of the time when the first symptoms were noticed it was found down in the pasture, and was killed and autopsied. This was a clear case of poisoning from the eating of *Aragallus lamberti*.

Case 8.—This steer was pastured on *Aragallus lamberti* from the first of the season of 1905, and no effects were noticed until the latter part of July, when it developed a solitary habit, and early in August was found to be very weak, stepping high and straddling with what are considered the peculiar loco motions. Plate III, figures 4 and 5, show this peculiarity of walking quite clearly. On August 27 the animal was down and unable to stand. When assisted to get upon its feet it would balance itself and fall over. Figure 6 of Plate III shows the animal in the act of attempting to stand after being assisted. At this time it would eat nothing, but would still drink a little water. This weakness came on very suddenly, for on August 26 it was driven to the pasture and came in in fairly good condition. The steer was killed on October 28 and the autopsy made.

Case 9.—This steer was fed on *Aragallus lamberti* from the beginning of the season. A note was made the latter part of May showing that the animal was in especially good condition at that time. At the end of June it was taken into the corral and fed cut *Astragalus mollissimus*. It ate freely at first but afterwards refused the plant, and an attempt was made to tempt it by mixing grain with the loco. It ate this for a little time, but soon would not eat the loco even with the chop, and because of the fear of starvation it was turned into the *Aragallus lamberti* pasture on July 21. At first the steer appeared to pick up and presented a very much better appearance. On August 30 it was taken into the corral again and fed with a mixture of *Astragalus mollissimus* and hay. It would eat the hay, but did not care for the loco. It was kept in the corral until September 17, during all this time eating the hay, but refusing the loco. As it was found impossible to make the animal eat the loco, the experiment of feeding

in the corral was abandoned, and it was turned into the loco pasture, where it commenced to eat *Aragallus lamberti* again. On October 14 it was noticed that the steer was very weak and would fall when driven rapidly. On October 27 it seemed best to kill the animal and make an autopsy.

Case 10.—This case was one of particular interest, as shown in the history of this and the succeeding years. The steer was put in the *Aragallus lamberti* pasture at the beginning of the season and on July 15 was taken to the Van Antwerp pasture with the hope of getting him to eat *Astragalus mollissimus*. This pasture at that time was filled with purple loco, and there was a considerable amount of *Aragallus lamberti* in the lane leading from the pasture to the corrals. There is no evidence that the steer ate any of the *Astragalus mollissimus*, but *Aragallus lamberti* was eaten by some of the animals and doubtless it had its share. Because of the destruction of the *Astragalus mollissimus*, on August 25 it was returned to the *Aragallus lamberti* pasture. The animal ate more or less of the *Aragallus lamberti* during the season, generally less, and as the season went on it ate less than in the earlier part of the year. On September 19 it was taken into the corrals and fed upon *Astragalus mollissimus* and hay. This diet was fed until October 8, but the steer continuously refused the *Astragalus mollissimus*. It was found impossible to force the loco diet and the animal was turned back into the *Aragallus lamberti* pasture on September 24. During the rest of the season it ate very little of the loco and at the end of the season was in good condition. This animal, it will be noticed, apparently ate the loco mainly because it was green and fresh and did not at any time acquire an appetite that would lead it to eat the plant in preference to grass.

Case 11.—This steer was placed in the *Aragallus lamberti* pasture at the opening of the season and ate loco from the start. No noticeable effect appeared until October. On the 16th of this month it fell when it was being driven into the pasture, and fell again at night as it was being brought in, showing that it had become extremely weak. At this time it was poor but by no means in a starving condition. It grew steadily worse, and on October 26 was found down and unable to rise. Its eyes were staring, its head shaking, and its coat rough. It was killed and an autopsy made October 27.

Case 12.—This was a black steer which was placed in the *Aragallus lamberti* pasture at the beginning of the season. This and No. 8 ate loco more freely than the other steers, and it was noticed that they were physicked more than was the case with the other animals. During the first part of the season both appeared to thrive upon the *Aragallus lamberti*. On May 30 this animal was taken into the corral and fed *Astragalus mollissimus* exclusively. At first it ate freely,

but later ate very little and finally refused absolutely to touch it and became very poor. On June 7 *Aragallus lamberti* was placed before the steer and it immediately commenced to eat it. Then the experiment was tried of mixing *Astragalus mollissimus* and *Aragallus lamberti*, and it was found that the animal would pick out the *Aragallus lamberti* and eat it but would leave all the *Astragalus mollissimus*. As it was evident that the animal would not acquire a taste for *Astragalus mollissimus* it was put on a diet of *Aragallus lamberti* in the corral. It was fed this exclusively from July 3 until July 24, but during this period was driven into the pasture on Sundays. In the pasture also it was noticed that it ate *Aragallus lamberti* very freely. The *Aragallus lamberti* was supplied to it in the corral in abundance and it ate very readily, but became steadily poorer, its coat becoming rough. During the last week of the corral treatment chop was mixed with the loco in order that it might not be without nutritive food, but on July 24 it was so much reduced that there seemed to be danger of starvation. Accordingly it was turned out into the pasture in the hope that it might pick up. Instead of picking up, however, it grew worse and became so weak that on August 1 it was found unwise to try to get it into the corrals. It steadily grew poorer and an autopsy was made on August 5.

EXPERIMENTS WITH HORSES.

Case 17.—This animal was an iron-gray horse, one of the best appearing animals of the lot received in 1905. Full of life and with a good gait, it was remarked by horsemen who examined the animals that he was an exceptionally good-looking animal. He was placed in the *Aragallus lamberti* pasture at the beginning of the season and presumably ate no loco at that time. On May 20 the horse was taken into the corral and fed upon cut *Aragallus lamberti*. He did not eat it readily at first, but afterwards ate it fairly well. On May 28 the horse was showing a very decided loss of flesh and his excess of life had disappeared. He had become a sleepy and dull horse. While he was being fed in the corral on June 6 he managed to get hold of a box of *Astragalus mollissimus* roots and devoured them very greedily. It was noticed, too, that when a wagon loaded with *Astragalus mollissimus* was driven into the corral he tried to get the loco from the sacks, although there was at the same time uneaten *Aragallus lamberti* in the corral. It was found at this time that he would make his way past numerous obstacles to get at bags of roots of *Astragalus mollissimus*. Although the way was barred, he would find the sacks and steal them. Our first impression was that he had a real passion for this loco. On June 8 the experiment was tried of mixing *Astragalus mollissimus* and *Aragallus lamberti*, when it was

found that he very carefully picked out the *mollissimus* and left the *lamberti*. He was then taken out to the pasture where both weeds were growing to see whether he would manifest the same choice, but there he devoted himself to grass, ignoring both kinds of loco. Later on, in thinking over the matter, the conclusion was reached that while he might have had some desire for the *Astragalus mollissimus*, the stealing of the roots and the cut loco might be explained simply as a desire to steal rather than a preference for the loco itself, because it would seem that if he really was anxious to get the loco he would have picked it out in the field, where there was an abundance of it.

The horse had become very poor at this time—July 18—and as it was deemed unwise to leave him in the corral he was put in the pasture and soon showed marked improvement. Up to this time there was no reason to think that he had eaten *Aragallus lamberti* in the field, but from now on he ate it quite freely. From August 2 to August 25 he was kept in the hospital pasture for fear of infection from glanders. Then he was returned to the *Aragallus lamberti* pasture and soon developed a solitary habit, grew exceedingly poor, and on September 20 was found in a dying condition.

Case 18.—This animal was kept in the *Aragallus lamberti* pasture from the beginning of the season until May 6, when it was taken into the corral and fed upon cut *Aragallus lamberti*. She ate it very freely almost from the start. From May 9 to May 21 she was kept in the pasture, and during this time apparently ate very little *Aragallus lamberti*. On the latter date she was again taken to the corral and fed with the cut weed. A week later, as she showed distinct evidence of having become poorer, and had a very sleepy appearance, she was put in the loco field for two or three days, where she ate *Aragallus lamberti* very freely. Then she was again taken into the corral and fed exclusively on *Aragallus lamberti*, except as she was allowed the run of the pasture on Sundays. She showed continuous loss of flesh until July 1, when she died very suddenly. During this time she had shown no peculiar nervous symptoms, but had simply gradually wasted away. The result of the experiment seemed to indicate that there was a lack of nutritive material in the plant, and that death was not only the result of poisoning, but probably the result of starvation. Plate IV, figures 1 and 2, show the condition of the animal after it had become locoed.

Case 19.—This mare was kept in the loco-free pasture until August 18. At that time she was taken into the corral and fed on *Astragalus mollissimus* and hay. She ate very freely in the corral, ordinarily picking out the loco from the hay, but also eating fairly well of hay. Later in the experiment there was a period when she picked out the hay rather than the loco, but most of the time she seemed to prefer



Case 8, July 19, 1905. Just before the locoed condition was particularly evident.



Case 8, August 3, 1905. When the animal showed typical symptoms of loco poisoning, especially in its attitude.



Case 8, August 23, 1905. Emaciated condition of animal and typical loco attitude of lowered head and braced legs.



Case 8, August 23, 1905. Loco leaping unnecessarily high in going over a rut in the road.



Case 8, August 23, 1905. Loco lifting foot unnecessarily high in passing over a wire.



Case 8, August 27, 1905. Animal too weak to stand unaided.

Mr. U



Case 18, May 12, 1905. A mare weakened by loco poisoning.



Case 18, June 27, 1905. Advanced condition of loco poisoning.



Case 19, May 16, 1905. Horse before eating any of the loco weed.



Case 19, August 31, 1905. Animal eating loco at a time when it had lost some flesh, but was not in bad condition.



Case 19, October 15, 1905. Animal in a very much reduced and emaciated condition.



Case 19, October 26, 1905. Animal just before its death.

4700



Case 524. Peculiar way in which a locoed horse uses its mouth in attempting to eat.



Case 533. How a locoed horse will rear when suddenly startled.



Case 525. Peculiar gait which a locoed horse exhibits.



Case 525. Locoed horse rearing when suddenly startled by a hat thrown out in front of it.



Case 529. A locoed Angora goat unable to get upon its feet, but otherwise fairly well.



Another attitude of Case 529.

1700

the loco. The experiment was continued until October 15, with the exception of Sundays, when she was allowed to run in the pasture. During this time she lost flesh very decidedly. From October 15 to October 18 she was kept in the *Aragallus lamberti* pasture to see if the freedom of the pasture would not cause her to pick up somewhat in strength and in flesh. She was brought into the corral on the morning of October 18 very poor and lifeless, dragging her hind feet as she walked, the near foot being more affected apparently than the off foot. It was decided to give her hay with chop for a few days to see if she could not be brought out of the diseased condition. She refused to eat the chop and on October 21 would eat neither chop nor oats, but would eat hay. She remained in the corrals until October 26, when she died and the autopsy was made. The death in this case was at least partly caused by starvation as well as by the effect of the poison. Plate IV, figures 3 to 6, show the change in the condition of the animal.

Case 20.—This was a yearling mare which was placed in the *Aragallus lamberti* pasture on May 8. She was not observed to be eating any loco until the latter part of August. After that time she ate more or less *Aragallus lamberti*. We do not know that the mare ate any large amount at any time, but she grew poorer steadily and disappeared on September 21, and was found dead a day or two later. The animal was interesting in that the poison took effect in a comparatively short time and rather unexpectedly.

Case 24.—No. 24 was a 6-year-old horse, a good-looking animal. He moved with erect head and long step, and was marked as one of the better appearing horses in the experiment. He remained in the loco-free pasture until the end of May and then was fed in the corral upon cut *Astragalus mollissimus*. He ate it very freely, in fact would eat about all that was furnished. This diet was continued until June 11, when the horse was turned out for a day. When returned to the corral he took the loco again very readily, although he did not seem hungry. There was no evidence that the horse ate any loco at all while in the field. At first there seemed to be no effect from this feeding experiment. The animal did not lose flesh, retained its spirits, and showed no symptoms of unhealthy nervousness. After the middle of June, however, he gradually lost flesh and early in July he was so poor that it seemed likely that we might have a case of starvation, so he was turned into the pasture to pick up. The animal still seemed to have fairly good spirits, carrying its head high, but the coat was rough and the bones exceedingly prominent. In the pasture he ate the grass, not caring for the loco, and it was hoped he would gradually pick up and get in form for further experimentation. Somewhat to our surprise on July 8 the horse was found dead. He lay

under a barbed wire fence and it was evident had kicked about for a considerable time before death. His death could not have been entirely the result of weakness.

It may be mentioned as a feature of this case that during the latter part of the horse's stay in the corral tumors as large as a small fist, one or two at a time, would appear upon the groin. These appeared and disappeared in a day or two. None were noticeable at the time of death.

Case 26.—This horse was taken from the loco-free pasture on July 13 to the Van Antwerp pasture to experiment with *Astragalus mollissimus*. There is no evidence that the animal ate any of the *Astragalus mollissimus* in this pasture, nor does it seem probable that it ate the *Aragallus lamberti*.

Because of the death of the purple loco the horse was brought back and placed in the *Aragallus lamberti* pasture on August 25. It began to eat the loco within two weeks, and soon it and its companion horse developed the solitary habit. The horse ate the weed continuously until September 15, when it died somewhat suddenly. The animal before this had shown a peculiar straddling gait which was especially noticeable in its hind legs, but it did not seem to be particularly weak nor in a bad condition.

CASES OF CATTLE AND HORSES NOT SUBJECTS OF EXPERIMENT.

Besides work upon the station animals a considerable number of locoed cattle and horses were examined at ranches near Hugo and at other points in Colorado. In most cases autopsies were made. Some of the more interesting cases will be briefly mentioned below.

Case 501.—This was a cow belonging to Mr. Frank Ewing, of Hugo. When found the animal was too badly decayed for autopsy, but it was a particularly interesting case of the very pronounced swelling under the jaw, which is considered as one of the peculiar symptoms of loco poisoning. The tumor was filled with a clear serous fluid.

Case 503.—This was a 2-year-old steer belonging to Mr. William Hazel, of Hugo. This case is interesting as being a typical locoed range steer. The animal was found near a watering place about 2 miles from the loco station. It was down, but with head erect. Its eyes were sunken and staring, its coat rough and very poor. When startled it responded with peculiar nervous twitchings.

Case 519.—This was the first horse upon which an autopsy was made. It belonged to Mr. Charles Johnson, of Akron, Colo., and had been considered one of his best horses. The animal had been eating loco for two or three years. It was poor in flesh, with shaggy mane and tail, its coat was rough, the hair being off in patches, and it walked with a peculiar stiff, irregular motion of the legs, and had become absolutely worthless.

Case 524.—This was a colt belonging to Mr. Kendrick, of Seibert, Colo. The interest connected with this animal was largely in its peculiar way of eating. It had been taken into the corral to feed in order to get rid of the loco symptoms. It was in good flesh, but very nervous, constantly walking about the corral. Plate V, figure 1, shows the peculiar nibbling way of eating, a motion that is characteristic of locoed animals.

Case 525.—This was a horse belonging to Mr. John Lieber, of Hugo, and is referred to in this place because of the particularly good pictures (Pl. V, figs. 3 and 4) which were obtained of its attitudes, which show the peculiar gait of a locoed animal and the attitude which one is likely to assume when startled. The horse was very poor, being little but skin and bones, with rough coat and shaggy mane and tail. When walking over a railroad track it was noticed that its feet were lifted very high in order to clear the rails.

Case 533.—In this case, too, a particularly good picture (Pl. V, fig. 2) was obtained of the position assumed when startled. This horse was seen on a ranch north of Claremont, Colo.

Case 529.—This case was one of considerable interest, as being the only case of a locoed goat encountered during the season's work. The animal was an Angora goat belonging to Mr. Lon Foote, of Hugo. It was received at the experiment station on September 23, 1905, and at that time was unable to stand. The animal moved its legs spasmodically, and by great effort, with assistance, would get upon its feet, but would soon fall down, falling clear over, with its head prone upon the ground. It would eat grain very freely and would drink readily. The first impression on seeing it as it lay upon the ground was that it was in the agonies of death, because of the peculiar convulsive movements of its legs. The owner said that it had been in the corral since spring. During the winter it had eaten very freely of *Aragallus lamberti*, and its present condition was considered as entirely due to this food. The goat was kept at the loco station from September 23 to September 29, and during that time showed very little change in condition, except that it grew somewhat weaker. Within two or three days after arrival at the ranch the goat was unable to stand upon its feet at all, and could not even sit with the head in an erect position for any length of time. When put in a sitting position so that it could eat, the goat would do so for a little time, then with a peculiar jerky, convulsive movement the head would be thrown back bit by bit, finally falling over on the side, with the horns lying flat on the ground. As it seemed probable that nothing more could be gained by keeping it, the goat was killed and autopsied. Plate V, figures 5 and 6, show the characteristic attitudes assumed by the animal.

SUMMARY OF FIRST SEASON'S WORK.

The detailed discussion of the results of the work of 1905 will be taken up after the description of the cases of the second year. It may be well, however, to insert here the statement which was formulated at the end of the season as embodying the results so far obtained.

1. There is no longer any question of the poisonous effect of the loco weeds. The results of the feeding experiment at the ranch seem to prove conclusively that *Aragallus lamberti*, or rattleweed, when eaten for a prolonged period of time, has an effect of poisoning upon the nervous system, which leads to a lack of muscular coordination. The animal's nervous system becomes impaired to such an extent that the nutritive functions are interfered with and the victim perishes by starvation. The presence of ulcers in the fourth stomach of some of the steers may possibly be a characteristic lesion. The finding of a serous exudate in the spinal canal in certain of the autopsies would seem to indicate that this is another definite lesion produced by loco.

2. *Aragallus lamberti* will evidently poison both horses and cattle; presumably also it has the same effect upon sheep, although the season's experiments did not have enough to do with sheep to make this at all evident.

3. *Astragalus mollissimus* has the same general effect upon horses and sheep as *Aragallus lamberti*. They do not eat it as readily, but when eaten it has the same general effect.

4. Cattle can be poisoned by *Astragalus mollissimus*, though as a matter of fact they rarely eat it. It was shown quite clearly by the feeding experiments that it was very difficult to make cattle eat this weed. In the neighborhood of Hugo, Colo., loco poisoning may be considered as almost entirely due to *Aragallus lamberti*. It is noticed, too, that in parts of the State where *Astragalus mollissimus* is the common loco plant, like the region around Holyoke, locoed cattle are almost entirely unheard of. The same may be said of the Panhandle in Texas, where the common loco plant is *Astragalus mollissimus* and where locoed cattle are very unusual. It seems probable, then, that the generalization may be made that, while both these plants will produce poisonous effects upon both cattle and horses, it is very rare that cattle will eat *Astragalus mollissimus*, and that so far as the cattle industry is concerned the loss from this plant is probably exceedingly small.

In the case of horses it appears that while they eat *Astragalus mollissimus* more readily than do cattle, they are not likely to eat it unless forced by shortness of food, while both horses and cattle may contract the habit of eating *Aragallus lamberti* even when an abundance of food is present.

5. It has been argued by some that the so-called loco disease was simply the result of starvation, and that if animals had sufficient to eat there would be no trouble from loco. The feeding experiments seem to indicate, however, a specific poisonous effect of the weeds. The animals that were fed upon loco alone starved to death, but animals were also given abundance of other feed mixed with loco, and in these cases they also died, showing conclusively that loco kills not simply by a lack of nutrition, but because of a definite poisonous effect produced by the plant itself.

At the end of the season, about the middle of November, the surviving animals were taken to a ranch for the winter, where they were pastured and fed hay when necessary, but none of them had any loco. It was intended that they should have the same care that would ordinarily be given by the stockmen to animals that were kept under common range conditions.

WORK OF THE SECOND AND THIRD SEASONS.

The work of the first season, as has been indicated above, seemed to bring certain fairly definite results. The experiment, however, was on too small a scale, the number of animals was too small, and the general character of the experiment too circumscribed to permit one to speak at all dogmatically as to the deductions which should be made. The autopsies in many cases were performed too hastily for satisfactory results. The labor of conducting the routine work was greater than had been expected and taxed the capacity of the station force seriously. It was therefore necessary to repeat the work of the first season on a larger scale if possible, and thus confirm or modify the tentative conclusions which had been reached. The plan of the second season (1906) then was on the same general lines as that of the first season, but modified somewhat by the experience already undergone. For example, there seemed no longer any doubt of the poisonous nature of the loco weeds, so that it seemed unnecessary to carry any large number of control animals. The season's work as planned involved:

1. Pasture feeding on *Aragallus lamberti* as in the preceding season.
2. Corral feeding on both *Astragalus mollissimus* and *Aragallus lamberti* under various conditions.
3. Careful post-mortem examinations to determine the definite lesions characteristic of the disease, the work of the first season not being conclusive on this point.
4. The determinations of the effects of loco on sheep as well as on horses and cattle.
5. Experimentation with such remedial measures as were indicated by the conclusions already reached.

6. The extension of the knowledge of loco plants as much as possible.

The preceding work and the main work of the second year were with *Astragalus mollissimus* and *Aragallus lamberti* in a restricted area. Not only should more be known of the extent of the damage from these two species, but it was desirable to know to what extent, if any, other related plants are the cause of similar results. At the conclusion of the second season it appeared that the first four heads had been covered fairly well, while a fair beginning had been made with the last two, although the work was incomplete.

In addition to the work at Hugo, in which the Colorado Agricultural Experiment Station cooperated as in the preceding season, the Department carried on a station at Woodland Park, Colo. Woodland Park is near Pike's Peak, and the conditions are very different from those in the plains, although the most abundant loco plant in this region is considered by botanists to be specifically identical with the *Aragallus lamberti* of the plains. Another loco plant, *Astragalus nitidus*, is abundant in the pastures in this neighborhood.

In cooperation with the Nebraska experiment station, a feeding experiment with *Astragalus mollissimus* was also carried on at Imperial, in western Nebraska. In this region the only abundant species of loco is *Astragalus mollissimus*.

The feeding experiment during the second season was carried on in the same general way as during the preceding year. Of the cattle, Nos. 1, 30, 31, 43, 46, and 47, and of the horses Nos. 49, 51, 54, 55, 61, and 65 were placed in the loco-free pasture as controls and kept there throughout the entire season. All prospered and were in good condition at the end of the season. Of the others, some were pastured in the *Aragallus lamberti* pasture and some were fed *Aragallus lamberti* and hay in the corrals. Twenty-three head of cattle were subjected to the feeding experiments, and of these eight were dead at the end of the season and two were locoed, but recovered under treatment. The history of some of the more interesting cases follows. In these cases, as in those of 1905, the results of the autopsies are discussed later on in this paper.

EXPERIMENTS WITH CATTLE.

Case 2.—This was a steer which had been kept in the loco-free pasture during the entire season of 1905 and during the season of 1906 until May 9. It was one of the best appearing animals, a particularly good looking and high spirited steer. On the latter date it was placed in the corral and fed on a mixture of hay and fresh cut *Aragallus lamberti*. At first it refused to eat the loco plant, and when the latter was mixed with the hay would shake out the loco weed and

eat the hay, but after a few days in the corral it ate the loco with a fair degree of readiness. The steer was kept in the corral until August 19, except for an occasional day, when it was permitted to run out in the *Aragallus lamberti* pasture. On May 27, when it was run out in the pasture it was noticed that it would eat nothing but *Aragallus lamberti*, and for some time before this date in the corral it had eaten loco rather than hay.

The first positive symptoms from the eating of *Aragallus lamberti* were noted on July 19, when it fell as it was passing through a gate to go to water. From this time on it always showed more or less symptoms of being gate shy; that is, would stoop in passing through a gate as though endeavoring to get under a wire. It became hard to drive and was more and more stupid as time went on. This stupidity was especially marked because of the prior activity of the animal. When kept in the loco-free pasture it had been the source of a good deal of trouble, because it was inclined to break through the fences and, when through, was caught only with difficulty. During the first week in August the animal lost its appetite and showed great nervousness, its head shaking as though it had the palsy. Sometimes in passing through a gate it not only would crouch but would fall down. Plate VII, figure 1, shows the peculiar attitude which it assumed at such times. It became very much constipated, and on August 12, when driven out of the pasture, would continually stumble and fall. It suddenly grew worse, and on August 23 fell and was unable to get up again. It died on the 26th and was autopsied.

Case 3.—This was another steer remaining from the season of 1905. It was in the loco pasture during that season, but did not show any marked results of poisoning. In the season of 1906 it was placed in the *Aragallus lamberti* pasture in the spring and immediately commenced to eat the weed. It ate very freely and began early to show the effect of the poison. On July 3 it was noticed standing by itself in the pasture, and on July 9 its condition was so marked that it seemed desirable to take it to the corral and attempt remedial measures. The steer was accordingly fed hay and chop, part of the hay being alfalfa. It gradually grew worse, however, and on July 16 was found down; in the afternoon it was deemed best to kill it and make an autopsy. Plate VI, figure 1, shows the animal just before its death, and Plate VI, figure 2, shows the interior of the wall of the fourth stomach, with the ulcers which were present in considerable numbers.

Case 10.—This was also an animal which had been experimented upon during the preceding season and had shown only the general effect of what may be called moderate eating. It was placed in the *Aragallus lamberti* pasture in the beginning of the spring of 1906 and ate the weed with considerable freedom. It was noticed toward the

end of April that the animal was very thin, its coat rough, and that it walked with its head down in a spiritless way. This probably was due not so much to the loco feeding as to the short feeding during the winter, as it had been in the loco pasture only a short time. From this time it commenced to eat *Aragallus lamberti*, but not very freely. It then weighed 507 pounds; on May 7 its weight was 590 pounds, and its maximum weight during the summer was reached on September 7, when it weighed 840 pounds.

The history of this animal was interesting because, while during the two seasons it ate more or less of the loco, it never ate it in any very large amount. It could well be compared to the moderate drinker of alcoholic liquors, for it was not poisoned so as to show the effect of the weed in a very marked degree, but it was affected to the extent of not gaining as much as would naturally be expected. The curve of the weight (see fig. 4) shows this effect, for if this curve is

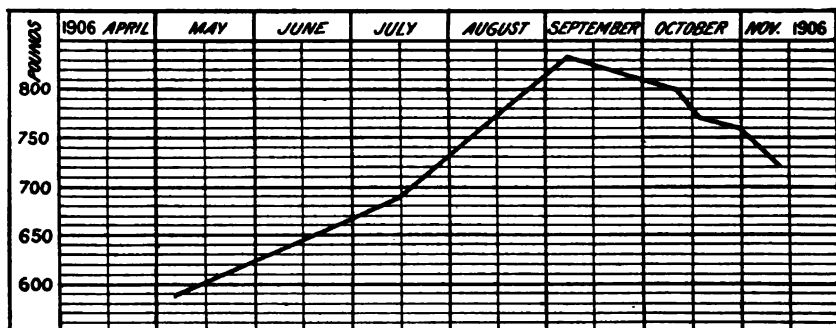


FIG. 4.—Curve of weight of steer No. 10, 1906.

compared with those of animals in good pasture it will be noticed that it is flatter, while the animals in good pasture gained with great rapidity until the end of the season. This was the only steer that refused to eat *Aragallus lamberti* in any large amount.

During the season of 1907 No. 10 was herded with the other experiment animals in the loco pasture and continued as in the preceding seasons to eat more or less of the *Aragallus lamberti*. At no time, however, did the steer eat enough to show marked effects from the poison. At the end of the season the gain in weight was once more much less than it should have been normally (see fig. 5), but this failure to increase in weight was the only evident effect of the loco poison. Case 10 is a good example of the possibilities of cattle eating loco season after season without showing serious effects from the poison.

Case 32.—This was an Aberdeen Angus cow carrying a registry tag. She was placed in the *Aragallus lamberti* pasture in April, 1906, and commenced immediately to eat the plant. She dropped a calf on May 6. She continued to eat *Aragallus lamberti*, and her



FIG. 1.—CASE 3. EFFECT OF LOCO POISONING, SHOWN IN EMACIATION AND DEJECTED ATTITUDE, JUST BEFORE DEATH OF ANIMAL.



FIG. 2.—INNER WALL OF FOURTH STOMACH OF CASE 3, SHOWING ULCERS UPON ITS SURFACE.

Wol



Case 2, July 25, 1906. Steer bending low to avoid an imaginary obstruction above it while passing through a gate.



Case 34. Another case similar to that in Fig. 1.



Case 67, May 1, 1906. A bright heifer calf shortly after birth.



Case 67, July 5, 1906. Calf shown in Fig. 3. Effects of loco which it had eaten in imitation of its mother.

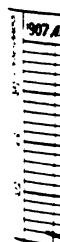


Case 67, October 16, 1906. Calf with typical symptoms of loco. The long hair of the face is especially noticeable.

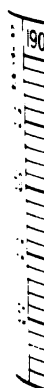


Case 67, October 19, 1906. Animal just before death.

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weight on July 5 was only 850 pounds as compared with 1,025 pounds on May 3. Of course her weight would have been reduced, in any case, at the time of the birth of the calf, but, under normal conditions, being a large, strong animal, the weight should have been regained in a short time.

The effect of loco upon her coat was noticed on July 19, although the symptoms were not at that time very pronounced. They became

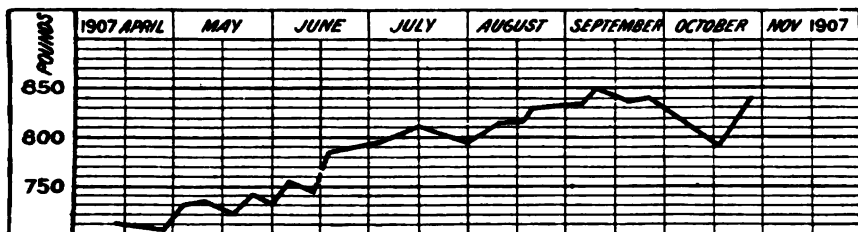


FIG. 5.—Curve of weight of steer No. 10, 1907.

more and more so, however, and on July 30 she had great difficulty in getting up after being down, and walked in the typical loco fashion. She died on August 26. The case was one of especial interest because she commenced to eat the loco almost immediately when out in the pasture, and the effects came in a comparatively short time. The effect of the poisoning on her weight is shown in figure 6.

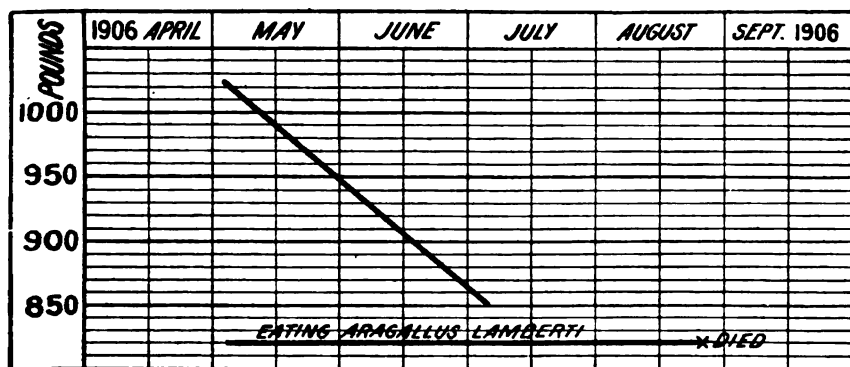


FIG. 6.—Curve of weight of cow No. 32.

Case 34.—This was a cow which commenced to eat loco immediately after being placed in the *Aragallus lamberti* pasture in the spring. She gave birth to a calf on May 3. The calf was weak and unable to stand and lived only a few days. There was really no reason, however, for supposing that the weak condition of the calf was due to *Aragallus lamberti*. She ate *Aragallus lamberti* with great freedom, and early in June it was noticed that at times she would go from

one plant to another, eating nothing at all except the loco weed. Symptoms of loco poison were shown in a marked degree a little before the middle of July. Plate VII, figure 2, shows the condition of the animal at this time, the picture being taken on July 9. It also shows the peculiar crouching attitude which the animal assumed in going through a gate—bending down as though she were attempting to get under a wire. On July 19 she was very poor, had a rough coat, straddled as she walked, and showed marked lack of muscular coordination; she was also very weak. She was kept on *Aragallus lamberti* until August 8. The cow was nervous, had lost control of her muscles to a very marked degree, and showed great difficulty in crossing over even the slightest obstructions. On August 8 she was kept in and fed alfalfa and hay, and attempts were

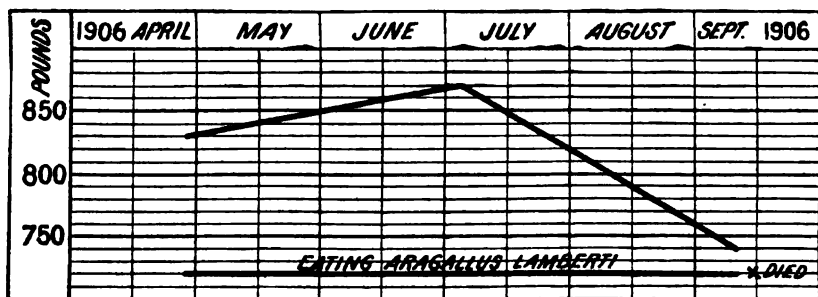


FIG. 7.—Curve of weight of cow No. 37.

made to give her certain medicinal remedies. She grew worse until August 16, when she died and was autopsied.

Case 37.—This was a Hereford cow with a registry tag. She was placed in the *Aragallus lamberti* pasture in the spring and immediately commenced to eat the loco weed. At this time she weighed 830 pounds. She gave birth to a calf April 28. She began to show the effect of the loco poison about the middle of July, and the symptoms increased gradually until August 31, when she would fall down when walking. On September 10 she was kept in the corral, as she was at this time very weak, and was fed hay. She weighed then 690 pounds. On September 11 she was down and remained down until September 14. During this time she would eat very little. On September 14 she was barely alive, and was killed in the afternoon and autopsied. The curve, figure 7, shows the decline in weight resulting from loco poisoning.

Case 45.—This was a cow which was placed in the loco-free pasture in the spring of 1906, weighing at this time 775 pounds. The latter part of May she was taken into the corral to feed fresh cut *Aragallus lamberti* in order to determine whether loco weed might not produce abortion. She was apparently about three months

along with calf. For the first two or three days the hay was omitted from her feed in order to get her to eat the *Aragallus lamberti* more freely. After that time, however, she was furnished with an abundance of hay so that there might be no question of starvation. She ate the weed from the start, although not very freely at first, but soon acquired the habit, and when turned out into the pasture it was noticed that she would go from one *Aragallus lamberti* plant to another, seeming to hunt for the loco, desiring it very much more than grass. On July 21 she was somewhat gate shy, and a few days later her motions in going through a gate very closely resembled those of steer 2. Her calf was dropped on August 5, and it was evident that it was a premature birth. The calf weighed only 14 pounds, was about 24 inches long, the hair was not formed, and it may be assumed that it was not more than six months along.

It would seem probable in this case that *Aragallus lamberti* had induced the premature birth. She continued to eat the *Aragallus lamberti* and gradually grew weaker little by little, losing her appetite and not caring to eat much of anything. On August 18 she was

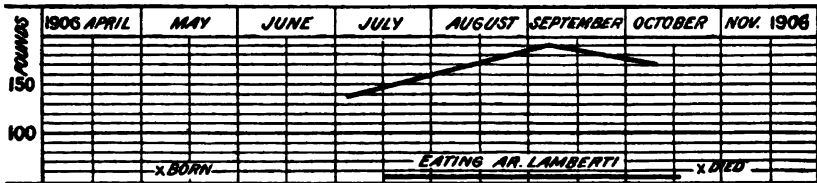


FIG. 8.--Curve of weight of calf No. 66.

down and could not get up. She was fed and furnished water, but did not get up again, and died on August 31.

Case 66.—This was a male calf dropped by cow 32 on May 6. Its weight on July 5 was 138 pounds. By the middle of July it was eating a good deal of *Aragallus lamberti*, and continued to eat it very freely; the effects of the poison were evident by the 1st of August. At that time the calf was decidedly constipated and began to be somewhat dull. On August 9 it was very weak and would not follow the others going to the pasture, but laid down and remained away from the rest of the herd. On September 7 its weight was 190 pounds. On October 9 the weight had become reduced to 170 pounds. On October 17 the animal was taken into the corral for dosing. It was pretty far gone, however, and in a blizzard on October 21 the exposure caused its death.

This case was particularly interesting, as the calf was born of a cow that was eating loco, and presumably learned to eat from its mother. Although a bright, healthy, and strong calf at the outset it made very little gain in weight and gradually succumbed. The curve, figure 8, shows the changes in weight.

Case 67.—This was a Hereford calf dropped by cow 37 on April 28. It was kept with its mother in the *Aragallus lamberti* pasture, and by the middle of June was eating pretty freely of the weed. On July 5 its weight was 160 pounds. Marked effects from the loco eating appeared about the 1st of August, when it became constipated and dull, and in other ways showed symptoms of the poison. The weight, however, increased until September 7, when it weighed 210 pounds. On September 19 it was put in good pasture, with the intention of

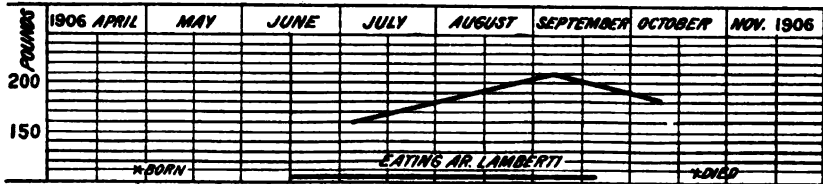


FIG. 9.—Curve of weight of calf No. 67.

making some attempt to cure its locoed condition. From this time on it was kept away from the loco and was dosed. It continually lost weight, however, and on October 9 weighed only 182½ pounds. Its decline was gradual, and it showed all the symptoms of typical loco. It became very poor, with rough coat, dull eyes, and in walking its head was down in an attitude of peculiar dejection. Plate VII, figure 3, shows the animal soon after birth, when it was a bright and handsome calf. Figure 5, taken October 16, shows the animal

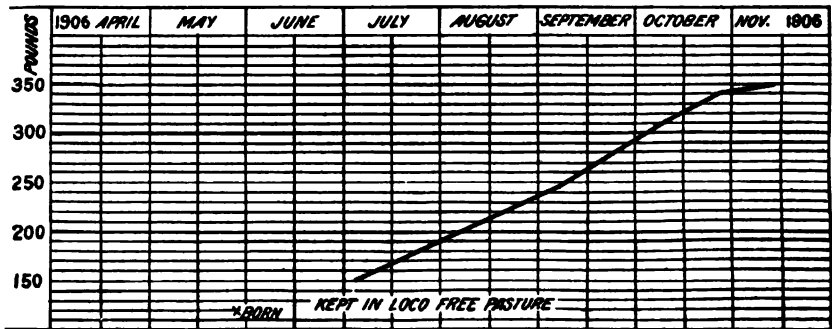


FIG. 10.—Curve of weight of calf No. 68.

when it had all the typical symptoms of loco poisoning. On October 18 it was down in the pasture, and remained down in the same position until its death on October 20. During this time an attempt was made to feed and water it, but without success. Figure 6 shows the attitude assumed by the animal during the last stages.

This was also a particularly interesting case, as the calf was born of a mother that was eating loco, learned the habit from its mother, and gradually succumbed to the disease.

It is of interest to compare this calf with another, No. 68, that was born about a month later. No. 68 was kept in the loco-free pasture through the whole summer. It soon outstripped No. 67 in weight, and at the end of the summer was a strong, healthy calf, weighing 350 pounds. The curves of weight of Nos. 67 and 68 are given in figures 9 and 10.

EXPERIMENTS WITH HORSES.

A few of the cases among the horses in 1906 deserve special notice as being typical of the general results of loco feeding.

Case 16.—This horse had been in the loco-free pasture during the season of 1905 and until June 11, 1906. From that time on it was fed *Astragalus mollissimus* and hay. At first the amount of hay was very small in order to induce it to eat more of the loco, but later on the amount of hay was increased, so that there might be no question in regard to the possibilities of starvation. The animal was kept

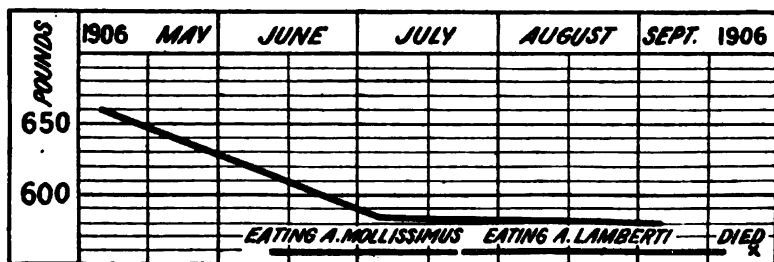


FIG. 11.—Curve of weight of horse No. 16.

upon this diet until July 21. During this time it lost in weight about 60 pounds. It was noticed that it ate the loco in a very variable manner; sometimes very little and at other times very freely. On July 22, as it had become impossible to obtain sufficient of the *Astragalus mollissimus*, the horse was put in the *Aragallus lamberti* pasture, remaining there until July 31, when it was taken in from the pasture and fed *Aragallus lamberti* and hay. This diet was kept up until September 19, when the horse was taken up for treatment, but it continued to grow worse and died September 25.

In this case there was decided trouble with the lungs as well as the ordinary loco symptoms. Plate VIII, figures 1 and 2, show the effect of the loco upon the animal. The curve, figure 11, shows the loss in weight.

Case 50.—This was an old horse that had evidently been worked for many years. It was received in the spring of 1906, and on arrival at the station was very poor, weighing only 670 pounds. It

was placed in the *Aragallus lamberti* pasture and immediately commenced to eat more or less of the loco plant. It gained somewhat in weight, weighing 695 pounds on July 18. Plate VIII, figure 5, shows its condition at this time. On September 7, however, it weighed only 660 pounds, having lost a good deal of flesh. It continued to eat *Aragallus lamberti* until September 16. On this date it disappeared from the station and was located several miles away, and the attempt to drive it in brought out the fact that it was not only poor but very badly locoed in every way. It was blind in one eye, probably having received the injury by running into a barbed-wire fence. It would walk directly into a fence, not stopping until actually cut by the wire. When driven, it would turn to one side and then move straight ahead, not stopping until it ran against a fence. In order to turn the animal it was necessary not only to get in front of it, but to strike it over the head. It was very poor at this time, but seemed to have a good deal of strength. On September 17 it was driven out,

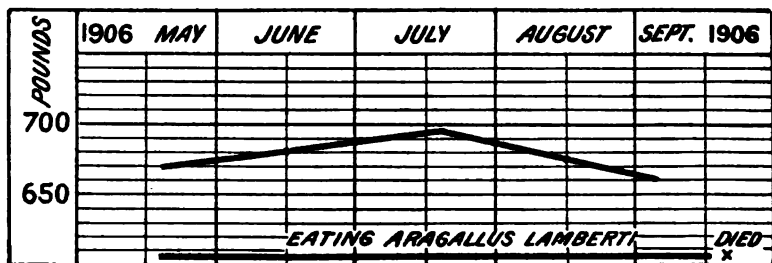


FIG. 12.—Curve of weight of horse No. 50.

but showed no sense of direction whatever in regard to the place it should go. Plate VIII, figure 6, shows its condition. The picture also shows very clearly the cuts upon its head which had been caused by running into a barbed-wire fence.

An attempt was made to treat the animal, but without any effect. On the morning of September 19 it was found dead. It had broken through three corral fences and had fallen down into a hole which had been dug for a cellar. Although the animal was old it was nevertheless strong, and in the early part of the season was in very good condition. Its end was doubtless due to the effect of the loco. Figure 12 gives the curve of its weight.

Case 52.—This was of interest as being one of the few cases of mules experimented upon. The animal was put in the *Aragallus lamberti* pasture early in the spring of 1906 and at first apparently ate none of the loco. Toward the end of May, however, some two weeks after it had been placed in the pasture, it was noticed that it not only ate *Aragallus lamberti* freely, but seemed to care for nothing

else. At this time it would pick off the leaves, but would not eat the flowers. It passed from one plant to another, eating them with great greediness. On June 18 it was weighed and found to weigh 590 pounds, a gain of 40 pounds from the time it was placed in the pasture. When weighed on September 7, heavy loss was shown, as it then weighed only 505 pounds. The mule was kept in the *Aragallus lamberti* pasture, but it was noticed on September 13 that it was not eating at all. Rather to our surprise, on the morning of September 15 it was found dead. It was thin, but nevertheless seemed to be quite strong. Plate VIII, figure 3, shows its condition in the spring, when the experiment commenced; figure 4, September 7, shows its peculiar attitude, with all the symptoms of loco poisoning very marked. Text figure 13 gives the curve of weight.

Case 59.—This mare was placed in the loco-free pasture during the early part of the season. She was about 8 years old and never had

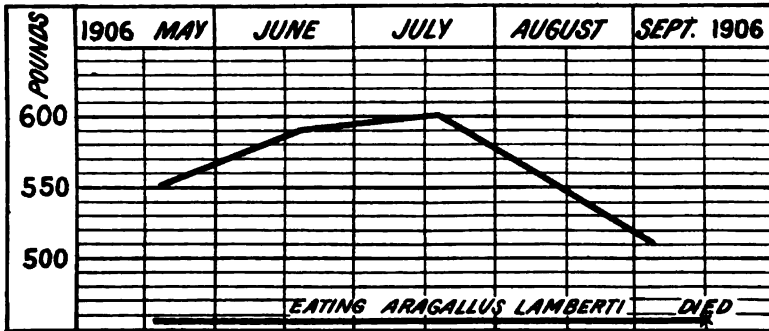


FIG. 13.—Curve of weight of horse No. 52.

been broken. She had a peculiar congenital malformation of the fetlock joints. Except for this defect, however, she was entirely sound and healthy. She increased in weight, and when, on July 18 she was placed in the *Aragallus lamberti* pasture she weighed 885 pounds. On September 7 she weighed 830 pounds. During this time she ate comparatively little of the *Aragallus lamberti*, and on September 18 she was taken into the corrals in order to hasten the effect of poisoning by forced feeding of *Aragallus lamberti* and hay. This feeding was continued until October 20, when for about a week she was in the *Aragallus lamberti* pasture. On the 28th it was noted that she was getting exceedingly poor and that the nervous symptoms were very marked. From that time on she was kept in the corral, fed hay and chop, and was treated, the treatment continuing through October and November with no apparent effect. This loss of flesh was particularly noticeable, because when she was taken into the corral to commence the feeding of loco she was an especially smooth,

handsome-looking animal, and the loco effect came very quickly and in a very marked way. She was taken to Fort Collins in December in order to continue the treatment during the winter, but died on the road. This was a typical loco case, the symptoms were marked and distinct, and the case is particularly interesting because the effect of the poison came in such a short time. Text figure 14 shows the curve of weight.

EXPERIMENTS WITH SHEEP.

Through the kindness of local sheep owners near Hugo considerable work on sheep was possible during the season of 1906. These sheep were numbered as received and were kept in the corral nights and herded in the neighborhood of the experimental ranch and on a pasture which was pretty well covered with *Aragallus lamberti*. Some of the sheep were very weak when received and in bad general condi-

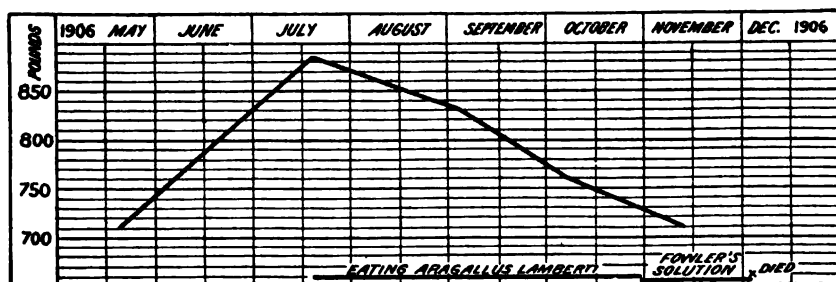


FIG. 14.—Curve of weight of horse No. 59.

tion. During the season 69 were used in the experiment, 63 being bucks a year old and 6 lambs. All were supposed to be locoed at the time when they were received.

In the early autopsies special attention was paid to the presence of parasites, particularly the grubs of *Estrus ovis*. The symptoms of this disease (grub in the head) are very similar to the symptoms of loco, and there was a possibility that the so-called locoed sheep were not locoed, but were affected by this parasite. *Thysanosoma actinoides* was also found in greater or less numbers in the duodenum or in the bile ducts, or in both.

Inasmuch as in the former investigations on locoed sheep under the direction of Doctor Marshall, a partial report of which was made in the Johns Hopkins Hospital bulletin, the very definite conclusion was reached that so-called locoed sheep were infested by these two parasites, and that if the parasites were removed the cause of the trouble would also be removed, these early autopsies were particularly interesting.



Case 16, June 6, 1906. Horse before eating loco weed.



Case 16, September 15, 1906. Animal shown in Fig. 1, in an advanced stage of loco poisoning, emaciated and extremely weak.



Case 52, May 19, 1906. Mule in fairly good condition before feeding upon loco weed.



Case 52, September 7, 1906. Mule shown in Fig. 3, in last stages of loco poisoning.



Case 50, July 19, 1906. An old horse before being fed with the loco weed.



Case 50, September 18, 1906. Same animal as in Fig. 5, showing effect of loco poisoning in attitude and emaciated condition; also shows cuts produced by running into barbed wire fence.

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A group of locoed sheep, showing the general appearance of locoed animals.



Sheep 27. A locoed sheep in last stages of poisoning.



Sheep 36. Effect of loco poisoning combined with grub in the head.



Same as Fig. 3, in a different attitude.



Case 66, October 20, 1906. A locoed lamb.



Case 66, November 12, 1906. Attitude of the animal just before its death.

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If the sheep were affected with loco poisoning it was evident that the effect of the loco was complicated by the parasites, and, of course, it might be possible that the primary trouble was not poison by loco plants, but the effect of these parasites. It therefore seemed desirable, if possible, to remove the parasites, and thus do away with the sources of complication. In this work of removal, however, the results proved that we were only partially successful.

Five of the sheep were given gasoline and milk as a vermifuge. Three of these were autopsied, with the result that one was found with many tapeworms, the other two with a few. It would appear, then, that the work of the vermifuge was only partially successful. It may have reduced the number of tapeworms, but it certainly did not clean them all out.

Four sheep were treated for *Æstrus ovis*. In two of these, holes were drilled into the frontal sinuses, and about a teaspoonful of gasoline injected into each side; they were then washed out with a fountain syringe with water containing a little gasoline. Of these two, one was operated upon on May 10 and died August 18, when there were still some individuals of *Æstrus ovis* in the frontal sinuses. The other was operated upon on May 9 and died the 21st, at which time 8 living grubs and several dead ones were taken out of the frontal sinuses and nostrils. Inasmuch as during the operation of washing out a considerable number of *Æstrus ovis* were seen to come from the nostrils, it is known that the operation was partially successful. It would appear, however, that in neither case did it entirely rid the animals of the grubs.

The other two cases were treated by injecting gasoline into the nostrils. In neither of these, unfortunately, were we able to make autopsies, so that the result of the experiment was not known.

A similar experiment was tried on some sheep in the season of 1907. Sheep Nos. 74, 77, and 80 were treated by injection of about a teaspoonful of gasoline into each nostril. One of these was later killed by a coyote. It was autopsied and no grubs were found in the frontal sinuses. Inasmuch as we feel certain of the diagnosis of grub, it would seem that in this case the treatment with gasoline was successful. Of the two others, No. 77 was in bad condition when treated, but immediately began to get better. No. 80 was also in better condition after treatment. It seems probable that in all these cases the gasoline treatment for *Æstrus ovis* was successful.

Soon after the first lot of bucks was received in 1906 an experiment was made to see whether they would eat loco readily or not. Hay mixed with *Aragallus lamberti* was placed in the corral where the sheep were confined. It was noticed that 12 of them would eat the weed to some extent. None of them ate very greedily, but

they would pass by the pile, perhaps pick up a single plant, and then pick up more hay. The others did not eat the plant at all. This preliminary experiment seemed to show that none were anxious to eat the loco and that many did not care to eat it at all. As the season went on, however, all of them ate more or less of the *Aragallus lamberti* in the field, sometimes eating a considerable amount and at other times only eating a little. Inasmuch as sheep have a way of nibbling at anything, it was sometimes rather difficult to tell whether they had a real passion for the weed or not. There were some, however, that would go from plant to plant and devote themselves for at least a portion of the day to eating nothing at all except *Aragallus lamberti*. It was also rather difficult to tell to what extent the animals were affected by the weed. Many of them were rather poor and weak. Some of them were more or less erratic in their movements, and as the season went on this became more pronounced. Instead of keeping together in a bunch as normal sheep do, they would scatter in groups of two or three, while sometimes a single animal would stray off by himself. It thus became increasingly difficult to care for them properly, as it was impossible to keep a herder with them all the time. Sometimes some of them would be lost for a day or two, and some disappeared never to return, having fallen prey to the coyotes.

Plate IX, figure 1, shows the general character of the sheep received from Mr. McIntyre in the spring. The cases all resembled each other very closely. Attention, perhaps, may be called to two or three of them, with the accompanying illustrations, as showing the typical condition of locoed sheep.

Sheep 27.—This was badly locoed when received and lived only a few days. Attention is called to it because the picture (Pl. IX, fig. 2) shows the typical appearance of a locoed sheep in the last stages of the disease.

Sheep 36.—This was interesting as being an animal upon which an operation was performed to rid it of *Æstrus ovis*. There seems no question that the operation was partially successful, although not completely so. The sheep was an old buck, perhaps 5 or 6 years old, and, except for his emaciation, was rather a handsome animal. He was kept about a month, when he died, his death without any doubt being the result of the loco poison. Plate IX, figures 3 and 4, show the condition of the animal.

Lamb 66.—In Plate IX, figures 5 and 6, are shown the peculiarities of locoed lambs. This was a lamb received in October, and was badly locoed. As the picture shows, the animal was in good condition so far as flesh was concerned. However, it was weak, staggering about in typical loco fashion. Plate IX, figure 6, shows the dull, dozy condition of the animal, the photograph having been taken only a short time before its death.

In 1907, besides a locoed wether presented by the Hamp Brothers, we had 15 bucks brought in by Mr. McIntyre on June 17. The history of these 15 bucks was of special interest because it differed so much from that of the sheep brought in by the same owner the preceding year. When they were delivered at the experiment station they were all poor, some of them staggering more or less as they walked, and they were considered by the owner and other sheepmen who saw them as typical specimens of locoed sheep. They resembled in their general appearance very closely the sheep that were used for experiment in 1906. From the very beginning, however, their behavior was different. While those of 1906 would feed where loco was abundant, confining their pasturage very largely to such localities, those of 1907 sought the lower land, where there was no loco but more grass, and even when the loco was abundant about them they paid very little attention to it. While the sheep of 1906 would scatter, going off in groups of two or three or individually, those of 1907 kept together and behaved more like normal animals. They were fed a little grain daily, mainly to accustom them to have a habit of coming to the corrals at night, but not enough to make any great difference in the formation of flesh. Nearly all of them gained through the summer, and were returned to the owner in good condition.

A few of these sheep, as explained elsewhere, were treated for *Estrus ovis*. Most of them had discharges from the nose, in many cases bloody, and it is possible that the real trouble with the band was not loco but the grubs of *Estrus ovis*. As is well known, animals generally recover from attacks of this grub, and it may be that the improvement of the sheep in question was due to the loss of the grubs in the course of the summer.

There is no doubt that nearly all of the sheep which were kept at the ranch in 1906 were locoed, and just as little that few if any of the band received in 1907 were affected by the loco poison. The experiments with sheep were, therefore, of particular interest as showing how easily the effects of grub in the head may be confused with loco poisoning. Incidentally, it would appear from our work that the injection of gasoline into the nostrils of sheep that are infested with the grub of *Estrus ovis* is likely to be followed with excellent results.

DISCUSSION OF THE SHEEP EXPERIMENTS.

Sixty-three of the 69 sheep used in the experiment of 1906 were bucks a year old or more, and 6 were lambs. All were supposed to be locoed when they were received. Autopsies were held upon 33 of the cases. In the autopsies special attention was paid to the presence of parasites because of the conclusions reached by Doctor Marshall, which have already been summarized in the discussion of the literature. Of the 33 on which autopsies were held, 23 had grubs of

Æstrus ovis. These were present in some cases in considerable numbers, and their presence was indicated before death by a bloody discharge from the nostrils. One case, No. 23, was considered to have died as the result of the presence of this grub. Twenty-three of the 33 were infected with *Thysanosoma actinioides*. In most cases, however, this tapeworm was not present in large numbers. In some the bile ducts were much distended with them, but commonly the numbers were rather small, and perhaps not more than would be expected in an animal which is so subject to parasitic infection. Cysticerci were found in 6 of the cases, but there is no reason to think that they affected the animals injuriously. In 13 of the post-mortem cases the inner walls of the fourth stomach were inflamed; 9 had blood clots in the lateral ventricles; 15 had an apparently abnormal number of hemolymph glands in the dorsal parts of the thorax, or the abdominal cavity, or both, and in 18 there was a serous coagulum present in the epidural space of the spinal canal.

In the experiment of 1907 most of the sheep were not seriously affected by the loco poison, but were suffering from other causes, the evidence tending to show that the presence of *Æstrus ovis* was in part at least responsible for their condition. The principal difficulty with most of the animals in 1906 was the loco poison, with the effects complicated by parasites. On the other hand, it seems that the chief trouble with most of the sheep in 1907 was caused by the parasites and that the loco had little if anything to do with their condition. It is evident from our work that it is very easy to confuse the effects of parasites with those of loco poisoning. The general appearance of the bands of sheep in 1906 and 1907 was the same, and not only the author, but experienced sheepmen, declared that both bands were locoed. In the majority of cases it was only by post-mortem examination that the diagnosis could be confirmed. These statements refer, of course, to the general symptoms. If the habits of the sheep are observed there is a marked difference. The sheep affected with *Æstrus ovis*, except when they are in very bad condition, keep together like normal animals, and show a preference for good food, although they may at times eat loco. The locoed sheep, on the other hand, are more erratic, and develop a solitary habit to a greater or less extent. They show, too, a marked fondness for the loco weed. At the same time, when one is dealing with a considerable number of sheep, it is a matter of much difficulty to separate the locoed animals from those affected with grub in the head. It is not strange that in experiments where sheep only have been used those in charge have been led to the belief that the so-called locoed sheep are sheep infested with parasites, for the symptoms bear a close resemblance. Moreover, it is very possible that in individual experiments the supposed locoed sheep were in fact not locoed at all.

It is a matter of considerable interest that lambs are much more quickly affected than older animals. Attention was first called to this fact by Mr. A. McIntyre, who stated that lambs would frequently succumb to the poison in two or three weeks, and with little loss of flesh. The author's observations were completely confirmatory of this statement. Of the 6 lambs under observation (Nos. 65 to 70), all were in good flesh, but very distinctly locoed. Post-mortems were held on 5 of these, and the results were very interesting. All had clots in the lateral ventricles. All had serous coagulum in the spinal canal, and all had congested walls of the fourth stomach. This would seem to confirm our opinion that these lesions are characteristic of the locoed condition, but that in chronic cases they may be more or less masked.

EXPERIMENT AT WOODLAND PARK, COLO.

The loco conditions were so different in the mountains that it seemed wise to conduct a feeding experiment to determine to what extent the phenomena would differ from those in the plains. The loco plants grow in the mountains to an elevation of 8,000 to 10,000 feet, and among the stockmen pasturing horses and cattle in these localities there are plenty of stories of locoed animals. The principal loco plant in the region where the experiment was carried on is *Aragallus lamberti*, but with this are associated a great many plants of *Astragalus nitidus* and a smaller number of *Astragalus splendens*. In the summer of 1906 land was offered for an experiment between Woodland Park and Divide, on the Colorado Midland Railroad. A particularly thick field of loco was fenced in for a loco pasture, and an adjoining piece with very little loco was used as a control pasture. Eleven horses and 12 head of cattle were used in this experiment, 6 of the cattle being donated by the Crescent Cattle Company, of Cripple Creek.

The horses were put in the pasture July 13—6 in the loco pasture and 5 in the control pasture. A week later 6 head of cattle were received and were divided, one-half being put in each pasture. On July 27 horses 73 and 74 were taken into the corral, where they were kept through the rest of the season, and fed on cut loco, with an abundance of hay. On August 14, 6 more cows were received and were divided between the two pastures. The cattle ate loco readily, and before the season was over cleaned out the pasture pretty thoroughly. The horses would not eat loco in the field, but those in the corral ate quite freely. None of the cattle showed any positive effects as the result of the loco eating. The horses in the corral grew thinner, became dull, and showed a somewhat stiffened gait. The lack of more general results in this feeding experiment was due in part probably to the comparatively short time during which the experiment was

carried on. It was unfortunate that we were unable to install the experiment earlier in the season.

The animals remaining in the fall of 1906 were taken to the ranch of the Crescent Cattle Company, where they were kept during the winter, receiving the ordinary treatment of the range stock owned by that company. During the winter horses 73 and 74 died, their death doubtless being occasioned by the weakness produced by the loco poison during the preceding summer.

In 1907, 8 horses and 8 cattle were pastured at Woodland Park; part of these, as in the preceding season, were kept in the loco pasture and part in another pasture comparatively free from loco. It was found, however, as the season progressed, that the so-called loco-free pasture had considerable of the plant in it, and some of the animals which were pastured in it became locoed in spite of the small temptation. As in the preceding season, we were unfortunate, because of a series of events entirely beyond our control, which made it impossible to start the real experiment until comparatively late.

The animals ate the loco readily, but none of the cattle were clearly locoed. Horse 76 was put in the loco pasture on June 11, and was badly locoed on August 6. This locoed condition was evident in her extremely nervous manner. She was easily startled and ate in a nervous way, as if frightened. She had the high step peculiar to locoes and the straddling attitude of the hind legs when she walked, and would rear when startled. At this time, however, there was no loss of flesh, but toward the end of August she showed a distinct loss of flesh, and on August 31 was taken up for treatment, the result of which is given elsewhere.

At the end of the season the stock was taken to Hugo to be pastured during the winter. At this time 5 of the 8 horses were distinctly locoed, but none of the cattle showed the effects of the poison.

The principal result of the two years' feeding at Woodland Park seemed to indicate that stock is not as easily locoed in the mountains as in the plains, and that horses are much more easily locoed than cattle. The latter fact seems to be in harmony with the results of experience, for there are very few complaints of locoed cattle in the mountains. A number of factors may explain the smaller number of locoed horses in the mountains. The loco is not so abundant as in the plains, commonly being restricted to smaller areas, while the grass is more abundant and commonly does not dry out, as it does on the plains, so that the animals are not forced upon the loco because of lack of other food. It is very possible, too, that the amount of the poison in the mountain loco is not as great as in the plants of the plains. This can only be determined by extensive laboratory experiments, for which thus far there has been no opportunity.

EXPERIMENT AT IMPERIAL, NEBR.

This experiment was carried on in cooperation with the experiment station of the University of Nebraska, which was represented in this work by Dr. A. T. Peters. The work was located at Imperial, in the southwestern part of Nebraska, and the animals for the experiment were donated by local stockmen. The loco in this section consists almost entirely of *Astragalus mollissimus*. The work was under the immediate supervision of Mr. L. B. Sturdevant, of the University of Nebraska.

In this feeding experiment the attempt was made to get results from the loco as rapidly as possible. With this end in view the animals were herded on the loco part of the time, and part of the time the weed was cut and fed to them. In the feeding experiments hay was always used as well as the loco, in order that there might be no question of starvation. The work was started about July 4 and was carried on to the end of October, when all the animals eating loco were dead. Only horses were used in this experiment, and the number was unfortunately much too small. All contracted the disease, however, and died with the typical symptoms of locoed animals, showing the same post-mortem phenomena as were exhibited in the animals studied in Colorado. Careful post-mortems were made, and the general results of the work served to confirm that carried on at Hugo. Thus the work in Colorado was supplemented in an important way, for it was impossible during the season of 1906 to obtain in the neighborhood of Hugo very much of the *Astragalus mollissimus* for feeding experiments.

REMEDIAL MEASURES.

In the work of the first season no attempt was made to experiment with remedies for the loco disease. The knowledge of the subject was so indefinite that there seemed to be no adequate foundation for such work. It seemed better to devote all our energies to getting a diagnosis of the disease and determining its cause before attempting to apply any remedies.

During the second season, however, it appeared that the correct diagnosis of the disease was gradually being evolved as the result of our observations, and it seemed best to make some tentative experiments with remedies. Inasmuch as most locoed animals are constipated, it was clear that something to produce free action of the bowels was of first importance. This would be true not only because it was desirable to have healthy action of the intestines, but because it would be presumed that a free action of the bowels would aid in eliminating whatever poison had been absorbed. The remedy used for this condition of constipation was magnesium sulphate.

When this was first used, the chemistry of the loco plant had not been worked out, and the reason for its use was simply to correct constipation. As it turned out later from the laboratory work, in this substance we were using the most probable antidote for the barium poison. This information, however, came too late to make it possible to experiment with antidotes. It would seem possible to arrange to give small doses of a sulphate which should neutralize the effects of the extremely small amounts of barium in the loco. Doctor True has suggested that this might be done in connection with salting the stock. It may be that through the food or drink it will be possible to give enough of an antidote, so that whatever effect the barium has may be nullified. Further experiments to this end will be made.

The effects of loco poisoning come on very slowly as a rule and after a long period of feeding. This period may not only be many days, but frequently months, and in some cases even years. The locoed condition seems to be the accumulated effect of a multitude of small doses of the poison, and the cure of the animals would most probably be sought in a treatment of the general condition of the animal rather than in any special treatment to counteract the effects of the poison. The most marked symptoms of locoed animals are the nervous phenomena and the anemia. These conditions were those that were especially considered in planning remedies. It seemed wise to pay more attention to building up the animals than to attempt to use an antidote. Several remedies were used which were clearly proved to be useless. One of these was potassium iodid, with which a fairly thorough trial was made. Asafetida, valerian, and caffein were tried, but with no good results. Strychnin seemed a logical remedy for the most evident symptoms, and was tried with a number of animals with varying results. Nine head of cattle, 3 horses, and 5 sheep were treated with this remedy. Most of those treated were in bad condition, and it was a fair question whether in any of these cases unfavorable results should not have been expected, or, rather, whether they would not have died in spite of all treatment. Two of the cattle made somewhat remarkable recoveries.

Two of the cattle were cured during the strychnin treatment and there seemed no good reason why this drug should not have the credit of the cure, but it was true that other animals treated with strychnin died, in some cases partly, at least, as the result of the poison. None of the horses showed improvement from the use of strychnin. The fact that several of the animals showed indications of poisoning made it probable that strychnin was administered in too large doses, although only the minimum doses of the ordinary veterinary materia medica were used. It seemed probable that locoes

perhaps were more sensitive to the effects of the drug and should be treated in especially small doses.

Arsenious acid effected a cure in one of the horses. This animal was treated at the agricultural experiment station during the winter of 1906-7 under the general direction of Doctor Glover, and while it was a clear case of loco in the fall of 1906 with all the typical symptoms, it came out in the spring definitely and apparently permanently cured. This animal was used as a saddle horse during the summer of 1907 and at no time showed any of the signs of loco poison, if we except a slight loss of spirits.

One of the cattle, too, after it was treated with Fowler's solution, was completely cured.

TREATMENT WITH STRYCHNIN IN 1906.

Case 4 was a steer that had been kept in the loco-free pasture during the season of 1905. In 1906 it was at first placed in the loco-free pasture, but on May 3 was put in the *Aragallus lamberti* pasture, where it commenced to eat the weed and continued to eat it with considerable freedom. Gradually the poison began to show its effect, and by the latter part of August the animal was recognized as a typical loco. It was in excellent condition at the beginning of the season, when turned into the *Aragallus lamberti* pasture. Plate X, figure 1, shows its condition May 30, 1905; figure 2, taken September 7, 1906, shows its condition on that date, when it was very distinctly under the influence of the poison. In this picture the attitude is that of the typical loco. It had lost flesh and showed nervous symptoms in the way of responding with convulsive movements to sudden sounds, and when made to run its head would shake with the palsied movement which is characteristic of many locoed cattle. In figure 3 may be seen the serous sac under the jaws, one of the typical symptoms of loco poisoning in cattle. The steer was taken up on September 20 for treatment and was given daily one-quarter grain of strychnin hypodermically until September 30, when the dose was increased to one-half grain. During this time it did not gain in weight, but the nervous phenomena gradually disappeared. The doses were kept up until October 6. From that time until the end of the season it was kept in the loco-free pasture. When it was sent away for the winter on November 20 it seemed to have entirely recovered so far as nervous symptoms were concerned. Figure 4 shows the animal's condition August 22, 1907.

The curve of weight for 1906 (text fig. 15) shows that this steer did not make normal gains. The loss for the end of October and for November, however, is the normal result for that time of the year. Storms and shortness of food caused losses that year in all the ani-

mals at that period. In the spring of 1907 the animal was kept in the loco-free pasture about one week. During the rest of the summer it was kept in the good pasture until September 18. From this time until October 28 it was herded on loco. As the curve shows (fig. 16), it gained during this time about 300 pounds. Plate X, figure 4, shows that on August 22, 1907, while in the loco-free pasture, the

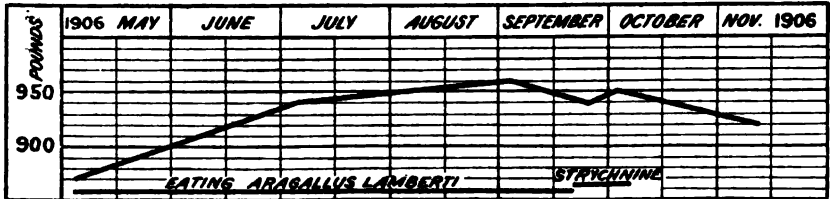


FIG. 15.—Curve of weight of steer No. 4, 1906.

animal was in fine condition. When it was kept on loco, however, it ate freely and at the close of the season it again showed distinct symptoms of loco poisoning, although it was not a bad case.

Another case, No. 536, was a Hereford steer belonging to Mr. Mattix, of Hugo, which was brought to the station for treatment on July 9. It was at this time extremely poor, its coat was rough, its

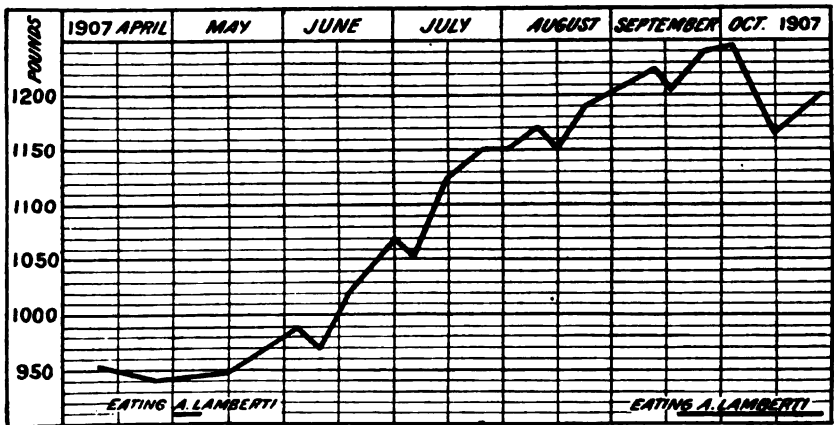


FIG. 16.—Curve of weight of steer No. 4, 1907.

eyes staring, and it stood with head low and legs spread apart because of evident weakness, and with a peculiar curvature of the fetlock joints of the hind feet which is sometimes noticed in locoed animals. Plate X, figure 5, shows very clearly the condition of the animal at this time. It appeared a hopeless case, because the animal not only showed the effects of poison, but was extremely weak. It was kept in the corrals, as it was too weak to be sent out into the pastures.



Case 4, May 30, 1905. Before feeding on loco weed.



Case 4, September 7, 1906. Showing all the typical symptoms of loco poisoning.



Case 4, September 15, 1906. Animal under influence of loco and with a noticeable accumulation of serous fluid under chin.



Case 4, August 22, 1907. Same animal as in Figs. 1 to 3, in the succeeding year, when it no longer showed symptoms of loco poisoning.



Case 536, July 9, 1906. A locoed steer with curvature of the fetlock joints, which is peculiar to some locoed animals.



Case 536, July 25, 1906. The same animal after treatment with strychnine. It stands in normal fashion and has gained much in strength and in weight.

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It was fed upon alfalfa hay and some grain, and was given hypodermically one-half grain of strychnin daily. The strychnin seemed to take effect upon it almost immediately and on July 11 its fetlock joints had straightened out and the animal showed itself in every way very much improved. The doses of strychnin were kept up from July 9 until July 21. Most of this time the action of the bowels had been very free, but as at this time it seemed somewhat constipated, it was given 250 grams of Epsom salts, and was turned into the loco-free pasture, where it was kept until July 30, when it was brought in and given another course of strychnin, one-half grain daily until August 6, when it was again turned into the loco-free pasture and was considered as very nearly cured. Plate X, figure 6, shows its improved condition on July 25. About October 1 it was turned back to the owner, who sent it out upon the range with his other cattle. The animal was seen again about the end of October and it was evident that the cure was complete and permanent. Later in the season the steer was put upon the market.

Case 40 was also treated with strychnin. This was a large, handsome steer which was received in the spring and kept in the loco-free pasture until July 18, when it was placed in the *Aragallus lamberti* pasture. It immediately commenced to eat the loco, and from this time on ate it with rather unusual freedom, seeming to prefer it to grass. The daily notes taken by the observers in the pasture indicate that it ate loco rather more freely than any of the other cattle. Its weight was 1,235 pounds when put in the *Aragallus lamberti* pasture. It gradually lost weight, and on October 16 weighed 1,160 pounds. Shortly after this the loco symptoms became very marked. The peculiar condition of the hind fetlock joints which was noticed in No. 536 was very marked in this animal also. At the end of October the steer was taken up for treatment. At this time it weighed 1,105 pounds. It was treated hypodermically with one-half grain strychnin daily and fed in the corrals with hay and chop. This treatment was continued until November 5. On November 12 its weight was 1,025 pounds. It was taken from Hugo to Fort Collins to continue treatment through the winter. From December 9 until December 20 it was given one-half grain of strychnin daily and showed a slight gain in weight. The treatment was then discontinued for a time, but was commenced again December 28 and continued in doses every second day until January 9. On January 31 it weighed 1,210 pounds. Two weeks later it had increased to 1,300 pounds. It was fed, of course, with considerable care, and on February 23 was sold for fat beef. It was at that time in fine condition. The curve, figure 17, shows the relation of the changes in weight to the feeding of loco and treatment.

During the season of 1906 none of the horses treated with strychnin showed any improvement, and the same can be said of the sheep. It may be stated that the doses of strychnin in the case of both horses and cattle were either one-quarter or one-half a grain, and in the case of the sheep from two-twentieths to eight-twentieths of a grain.

There was no question that in some cases there were symptoms of strychnin poisoning, and this was true of cattle, horses, and sheep. It seemed probable that the doses of strychnin were too large, so that in the weakened nervous condition of the animals they were affected unfavorably. It appears that locoed cattle may be unusually susceptible to the influence of strychnin. At the same time the extremely favorable results in the cases of Nos. 4, 536, and 40 made it seem desirable to make further experiments with strychnin, but with the use of much smaller doses.

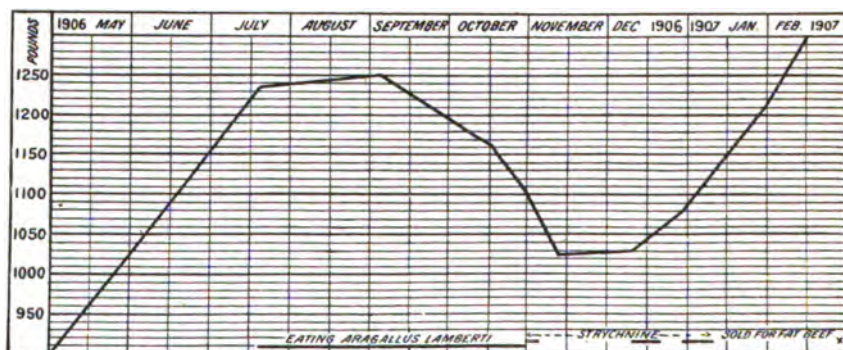


FIG. 17.—Curve of weight of steer No. 40.

TREATMENT WITH ARSENIC IN 1906.

Arsenic either in the form of Fowler's solution or of arsenious acid was used with 3 horses, 4 cattle, and 4 sheep. Two of the horses died, one (No. 58), a mare, was cured. The history of this case is given in some detail.

The mare was received from the Fort Collins Agricultural Experiment Station on May 18, and was placed in the loco-free pasture. Her weight at this time was 655 pounds. She had been an old saddle horse, was a lively, spirited animal, although not young, and in June was used as one of the saddle horses at the station. It was found, however, that her nervous disposition made her somewhat undesirable for general work, and on July 30 she was put in the *Aragallus lamberti* pasture and kept there until October 9. During this time there is no evidence that she ate any of the loco weed. Apparently she devoted herself entirely to grass. On October 9 she was taken into the corral and fed cut *Aragallus lamberti* and hay. At this time

she weighed 765 pounds. She was kept during the remainder of the season upon this treatment.

After October 16 the mare gradually lost in weight and on November 12 weighed only 705 pounds. At this time she was weak and in her peculiar nervous actions showed very decided loco symptoms. She would start at sudden noises and would rear when any sudden motion was made near by. She was taken to Fort Collins for treatment during the winter, under the general direction of Doctor Glover. It was reported from there on December 14 that she was very badly locoed. She reared when excited, and could not be led or tied. This in spite of the fact that she was a so-called gentle horse and had been used for several years. On December 16 treatment was commenced by giving her $2\frac{1}{2}$ grains of arsenious acid in chop. This treatment was continued until January 8. On January 9 she weighed 805 pounds, which was a very marked gain, as on December 12 she weighed only 740. On March 1 she weighed 865 pounds. It was reported at this

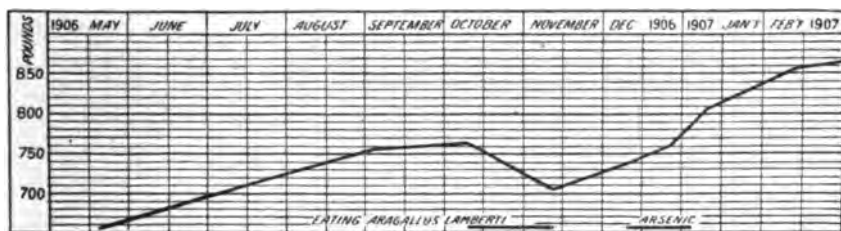


FIG. 18.—Curve of weight of horse No. 58.

time that the man in charge of her tried to ride her, but could not manage her well, and that she would not lead. Her legs were slightly swollen on this date, probably from an overdose of arsenic. No further treatment was given beyond good feed, and she was taken to Hugo in the middle of April and placed in the loco-free pasture. For about a week in May she was herded upon *Aragallus lamberti* and ate it with some freedom. She was then put back in the loco-free pasture, having shown no symptoms of poisoning, and a little later, May 10, was again used as a saddle horse. It was found that she was entirely reliable and showed none of the symptoms of loco poisoning, if we except a slight lack of spirit. She was used during the whole summer in the general work of the station as a saddle horse and did the work well. This was an evident case of cure and presumably the arsenic was instrumental in effecting it. Unfortunately we have no good pictures showing the progress of the disease and cure of this animal. The curve (fig. 18) shows her changes in weight while under treatment.

Of the horses that died while being treated with arsenic, one was a very bad case and probably could not have been saved by any

method of treatment. The other was a good case for treatment, but died in spite of all that was done for her. This case (No. 59) became worse very rapidly, there being only eighteen days of treatment with Fowler's solution before the animal died. It may be considered, perhaps, as a case of rather acute poisoning.

Of the sheep treated with Fowler's solution, all but one died, and we did not feel at all certain that the cure of this one was due more to the Fowler's solution than to the effect of good food.

Of the cattle treated with Fowler's solution, all but one died. The one cured (No. 28) was an old milch cow which was received from Denver and at first placed in the loco-free pasture. She was kept there until June 18, when she was brought into the corrals and fed on *Astragalus mollissimus*. At that time she was heavy with calf, and inasmuch as the loco weeds are popularly supposed to produce

abortion, it was desired to feed this animal loco to see whether any effect would be produced on the birth of the calf. She was fed in the corral from June 18 until July 22, with the exception of occasional days in the loco pasture, her rations consisting of fresh-cut *Astragalus mollissimus* and hay.



FIG. 19.—Case 28, August 27, 1906. Cow after it has become locoed, but not a bad case. The serous sac under the chin is very prominent.

She ate both with very great freedom and showed a strong appetite for the loco weed. By the end of July it was necessary to terminate the experiment with the *Astragalus mollissimus* because it was found impossible to obtain enough for feeding purposes, and on August 2 we commenced feeding her hay and fresh-cut *Aragallus lamberti*. It was noted on August 7 that she showed no effects from the loco feeding, but on the 14th the effects began to appear. She dropped her calf on August 20. Her calf was small and weak, but was fully matured. Whether its weakness could be considered as the effect of the loco is a matter of doubt. The cow was kept in the corral and fed *Aragallus lamberti* and hay until September 17. During this time the effect of the loco poison became more and more evident. Figure 19 shows the condition of the animal on August 27. At this time the serous sac under the jaw, typical of many locoed animals, was very prominent. She was put in the loco-free pasture on September 18 and

kept there with no further treatment until October 31, in order to see whether simply having good feed and being taken away from the loco might not bring about her recovery. During this time she had gained slightly in weight at first and then lost. On November 3 we commenced treating her with 15 c. c. of Fowler's solution daily. This treatment was continued until the end of the month, when she was taken to Fort Collins for further treatment during the winter. During the latter part of December she was treated with potassium iodid, but with no particularly good effect. On February 1 treatment with Fowler's solution was resumed and she received 15 c. c. daily through the month. Later the treatment was discontinued. About the middle of March she was shedding her winter coat and getting fat, and the last of March she was fat enough for beef.

This was evidently a cure, and the credit of the cure must be given to Fowler's solution. The after history of No. 28 is interesting, as she was kept during the summer of 1907 upon a pasture

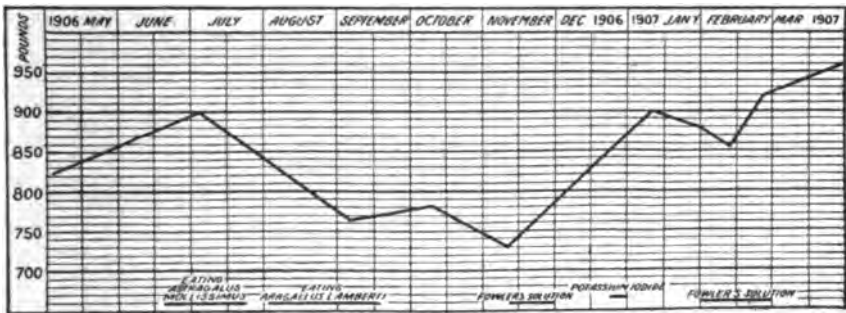


FIG. 20.—Curve of weight of cow No. 28, 1906.

where loco was very abundant and ate more or less of it through the season, but without any noticeably deleterious effects. The curves (figs. 20 and 21) show the changes in weight in the two seasons in their relation to the treatment.

RESULTS OF TREATMENT IN 1906.

In summing up the general results of the remedial measures attempted in 1906, it seemed quite clear that of all the remedies with which experiments were made success had been reached with only two—strychnin and Fowler's solution. The marked cases of cure in cattle with strychnin seemed to make it very probable that this remedy would prove useful. It also was evident from the deaths which occurred from the use of strychnin that it would be necessary to use very much smaller doses.

The results from Fowler's solution were somewhat doubtful. We have the cases of horse No. 58 and of cow No. 28, which presumably were aided in their recovery by the use of the solution.

Simply a statement of the number of cases in which there was a cure or a failure to cure would hardly give a fair idea of the results of these experiments, but in each case the individual peculiarities of the patient should be considered. As a matter of fact, in most of the cases which were subjected to treatment in 1906 the disease was very far advanced, so that the chances of cure with any course of treatment were very small. This was true in nearly all the cases of sheep and in most of the horses and cattle. The recoveries came only after a somewhat prolonged course of treatment. One of the cattle which recovered was treated forty days, another twenty-seven days. A horse which recovered on a treatment of arsenious acid was treated for thirty days. From the summer's work it seemed wise to experiment further with strychnin and arsenic,

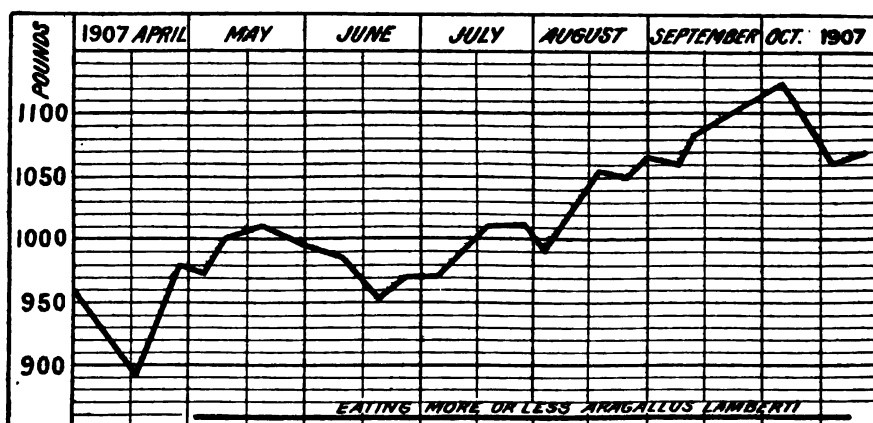


FIG. 21.—Curve of weight of cow No. 28, 1907.

using the former mainly with cattle and the latter for the most part with horses.

During the summer of 1907, then, it was determined to make as fair a trial as possible of strychnin and arsenic, using smaller doses of the strychnin. Inasmuch as some success had been obtained with arsenious acid and Fowler's solution, experiments were made with atoxyl and sodium cacodylate, it being thought that other forms of arsenic might have the same good effects. It may be remarked that the pathological lesions of locoed animals are surprisingly like those produced by the trypanosomes, and, granted that the use of atoxyl in trypanosomiasis succeeds not only because of its specific effects on the parasites but also because of its effect on the system generally, it might be possible that in atoxyl there would be a remedy also for the condition found in locoes. Sodium cacodylate was used because of the marked benefits sometimes produced in cases of anemia in human patients.

TREATMENT OF CATTLE IN 1907.

Fowler's solution.—Only one of the cattle (No. 565) was treated with Fowler's solution. It was a typical loco, although not extremely poor, and the nervous symptoms were not as pronounced as in some cases. The solution was given in 15 c. c. doses and was continued for fifty-three days. The steer gained, but quite slowly. Its weight at the beginning of treatment was 580 pounds, and when it was discharged it weighed 670 pounds. It was considered a cure, but the results of Fowler's solution did not seem to be as marked as those that were obtained with strychnin.

Atoxyl.—Atoxyl was used in three cases—Nos. 42, 546, and 558. No. 42 was one of the station animals that had eaten loco during a considerable portion of the preceding season, but did not show marked symptoms of loco poisoning. This steer was herded upon loco from the beginning of the season in 1907 until the 1st of August. As early as July 4 it was noted that the animal was holding its head low in the typical loco fashion and that the movements of its legs were somewhat nervous and jerky. A few days later the steer had the staring look in the eyes which is seen in so many locoes and the bending of the fetlock joints of the hind legs. It was kept, however, on the loco until August 1, when it was taken in for treatment. At that time the loco symptoms were very pronounced and the animal was quite weak. It was given 0.28 gram atoxyl and this amount was increased in succeeding days until a maximum of 1.4 grams was reached. This was given until September 2. When the treatment commenced, on August 2, the steer weighed 840 pounds. At the conclusion of the course of treatment the weight was 888 pounds. From that time the animal was kept in the loco-free pasture and gradually increased in weight until the end of October, when it weighed 960 pounds. Before treatment it had the typical dejected attitude of the loco and the peculiar flexing of the fetlock joints of the hind legs, but recovered so that it was bright and walked in normal fashion. This case can be considered as a cure, and the credit of the cure probably can be given to atoxyl. The curves (figs. 22 and 23) show the changes in weight in the history of the animal.

No. 546 was treated for a time with atoxyl and afterwards with strychnin, as there seemed to be no gain from the atoxyl. The gain under strychnin in this case was slight.

The third case which was treated with atoxyl (No. 558) was a bad case from the start, and probably no remedy would have succeeded in bringing the animal out.

Sodium cacodylate.—Five of the cattle were treated with sodium cacodylate, and 4 of these (Nos. 3, 6, 35, and 548) were cured. The one not cured (No. 559) was in the last stages of the disease and probably beyond any cure.

No. 35 was a cow belonging to the experiment station which had been eating *Aragallus lamberti* during the season of 1906, but did not seem to be affected by the poison. During 1907 she was herded upon loco from the first of the season and ate the weed quite freely. On July 1 she was taken in for treatment, as at this time she showed very

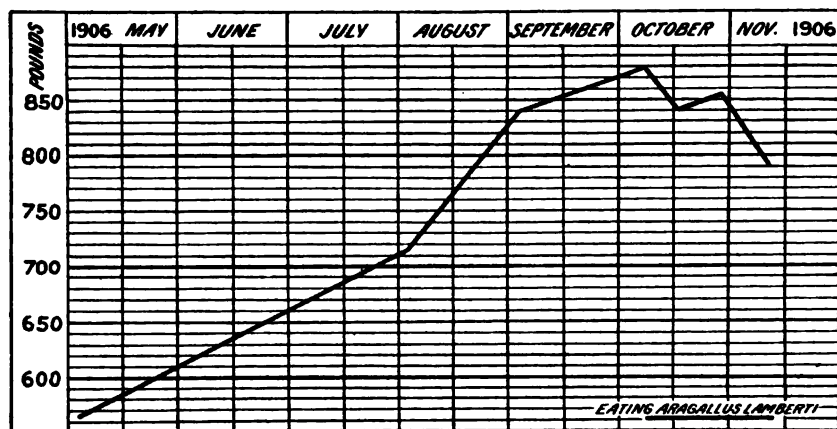


FIG. 22.—Curve of weight of steer No. 42, 1906.

distinct signs of being locoed. For a considerable time before this she had been constantly losing weight. She was given 0.6 gram of sodium cacodylate hypodermically. This treatment was continued from July 1 to August 10. Her weight just before the treatment commenced was 792½ pounds. At the time the treatment ceased she

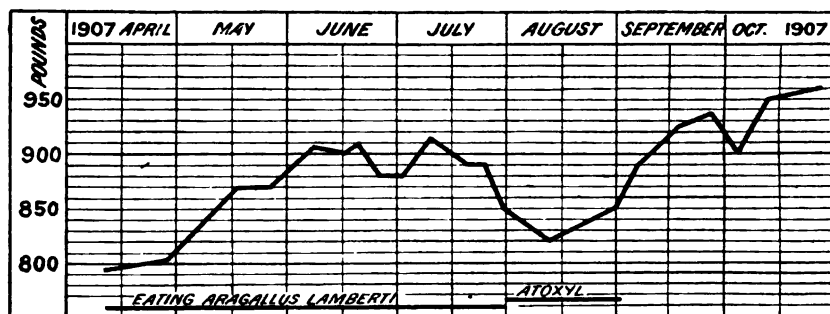


FIG. 23.—Curve of weight of steer No. 42, 1907.

weighed 882½ pounds. From this time she was kept in the loco-free pasture and at the end of October weighed 990 pounds (see fig. 24).

No. 548 was a heifer belonging to Mr. Woods, of Hugo. She was brought to the station from the Woods ranch on April 22. She was very thin and wild eyed and when eating showed in a marked way the peculiar stiff action of the jaws which is frequently characteristic

of locoed animals. She was given a few doses of magnesium sulphate and three-twentieths of a grain of strychnin was administered hypodermically from April 23 to April 27. From this date sodium cacodylate was given hypodermically in 0.3-gram doses, and the treatment was continued until May 13. The dose was increased to 0.4 gram

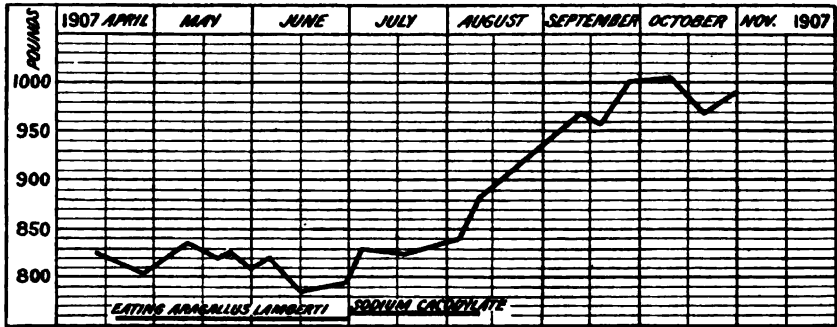


FIG. 24.—Curve of weight of cow No. 35.

from May 14 until May 31. From June 1 until June 15 daily doses of three-twentieths grain strychnin were given. On June 16 she was put in the loco-free pasture and the treatment was stopped. Her weight at the beginning of the season was 440 pounds. At the time treatment was discontinued she weighed 530 pounds and gained

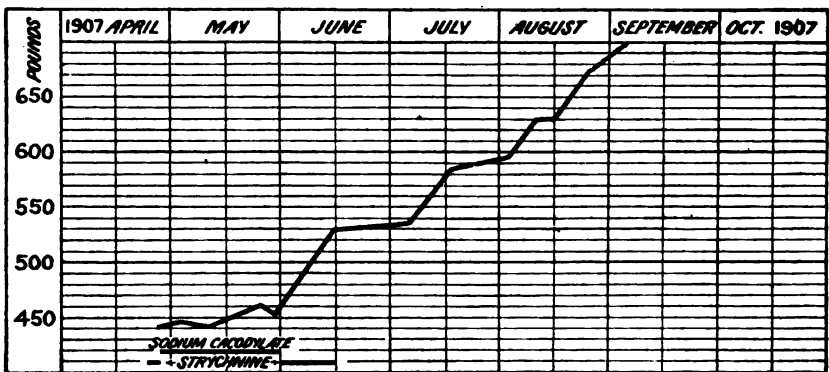


FIG. 25.—Curve of weight of helper No. 548.

continually from that time on (see text fig. 25). She was kept in the loco-free pasture until the middle of September, when she weighed 702½ pounds. The gain of this animal was very marked and the change from a poor, worthless animal in the spring into an animal fit for beef in midsummer was very noticeable.

No. 556 was a steer received from Mr. Mosher, of Hugo. It was one of the wild locoes and very difficult to drive. It was extremely

excitable when first brought to the station. Its coat was rough and the animal was quite thin. We commenced by giving this animal three-twentieths grain strychnin doses, but this was continued for only four days, after which it was treated with one-half gram sodium cacodylate, given hypodermically. This treatment was continued from July 14 to August 23. When treatment commenced, the steer weighed $717\frac{1}{2}$ pounds. At the time treatment was discontinued its weight was 810 pounds. It was kept until the middle of October, at which time it weighed 925 pounds.

Strychnin.—Fourteen head of cattle in 1907 were treated with strychnin. Of these, Nos. 503, 559, 561, and 566 died. No. 559, when received, was very poor and weak, and lived a comparatively short time. No. 566 was so weak that a few days after it was received it fell near a creek with its nose under water and was drowned. No. 561 had a complication of troubles that led to its death, although the treatment with strychnin was evidently beneficial. It gained in weight and apparently was doing better, but died, as the autopsy showed, of pericarditis.

Nine of the animals treated were distinct cures. Of these, No. 38 had been kept in the loco pasture during the months of October and November in 1906, but without showing any effect of loco poisoning. In 1907 it was herded on *Aragallus lamberti* from the beginning of the season until July 1, when it was taken in for treatment. For some little time before this it had been showing distinct loco symptoms, carrying its head low, and being slow in its movements. It showed more or less lack of muscular coordination in its limbs. A few days before this, on June 27, it had broken a horn and it became necessary to cut it off. It lost considerable blood in this operation, and this may have somewhat increased its inactivity. It was given four-twentieths grain of strychnin for five days, when the dose was reduced to three-twentieths grain. This was continued until July 31. During this time it became extremely weak—so weak that it could not be driven to the stock yards for weighing, or even taken out into the good pasture, but was kept near the house in the horse pasture. It began to pick up in about a couple of weeks, and on July 24 was taken out into the loco-free pasture with the other animals. Its weight on July 26 was 700 pounds. From July 31 it was given four-twentieths grain of strychnin daily, and this was continued until August 23, when it weighed 760 pounds. It was kept in the loco-free pasture during the remainder of the season and on November 1 weighed 875 pounds. It was then considered as entirely cured. The curve of weight is shown in text figure 26.

No. 547 was a heifer received from Mr. Woods, of Hugo. It was brought to the station April 22. It was a yearling heifer, very thin and wild eyed, and dragged its hind legs when walking, in the typical

loco fashion, and it was noticed that it ate with the stiff motion of the jaws which is peculiar to locoes. The animal was treated by administering three-twentieths grain strychnin hypodermically daily until May 14, when the dose was increased to four-twentieths grain. This treatment was continued until May 25. Besides the treatment with

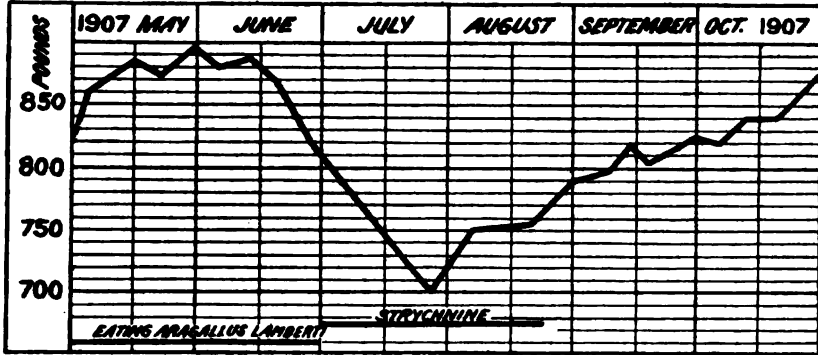


FIG. 26.—Curve of weight of steer No. 38.

strychnin the heifer was given several doses of Epsom salts in order to insure free movement of the bowels. From April 25 she was kept in the loco-free pasture until the latter part of the season. When received, she weighed 465 pounds; on May 24, when treatment was

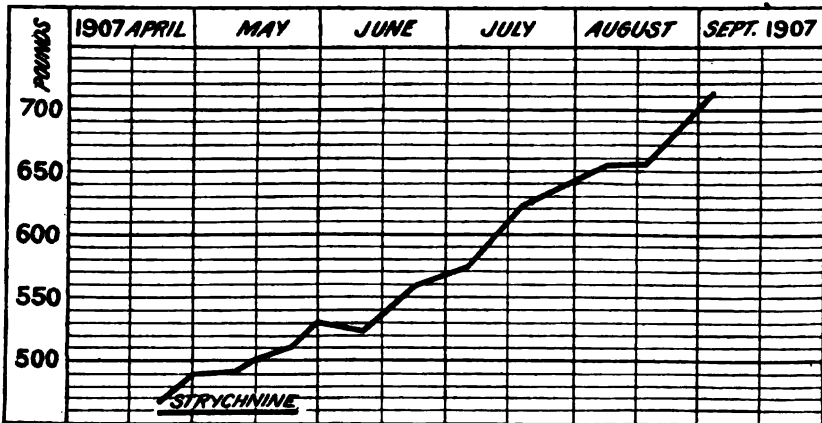


FIG. 27.—Curve of weight of heifer No. 547.

discontinued, her weight was 510 pounds, and it continued to increase until the middle of September, when she weighed 712½ pounds.

Plate XI, figures 1 and 2, show the animal's condition on April 23 and August 22. On June 4 the heifer was practically a cured animal and was in thoroughly good shape, while at the date of the last picture, August 22, she was in condition fit for market. The curve (fig. 27) shows the increase in weight.

No. 555 was a steer received from Mr. Mosher, of Hugo, on July 9. It was weak, with rough coat, in poor flesh, and quite wild. Treatment was commenced on July 10 with three-twentieths of a grain strychnin daily and continued until August 23. During this time it had gained 60 pounds. Subsequently it was kept in the loco-free pasture until it was returned to the owner about the middle of October. At that time it weighed 750 pounds and was considered a complete cure. The curve (fig. 28) shows its increase in weight.

Two other cattle were treated with strychnin. One of them, No. 68, was treated for too short a time to show any marked effects, although it was noticed that there was some improvement. The other one, No. 569, showed improvement, but had other troubles which prevented a complete cure.

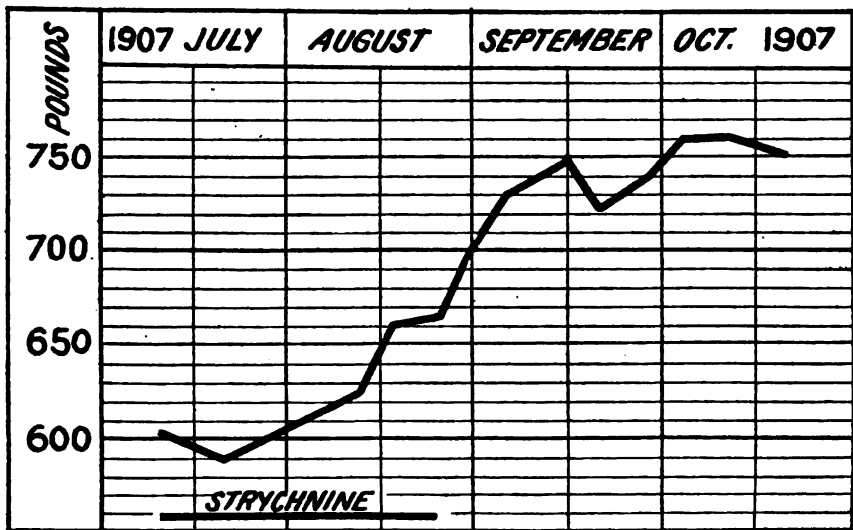


FIG. 28.—Curve of weight of steer No. 555.

TREATMENT OF HORSES IN 1907.

During the season of 1907 seven horses and one mule were treated. Strychnin was used in only one case, and in this case not only the strychnin was used, but also sodium cacodylate. It is difficult to tell just what the effect of either of these substances was upon the horse treated (No. 22). It gained during the season, but gained somewhat slowly and could not be considered as a cure.^a

The other seven animals were treated with Fowler's solution alone. Of these, two were distinct cures, and the others with one exception all made gains. The exception was the mule. This was an old

^aNo. 22 was pastured during the season of 1908 with no further treatment and became a healthy, handsome animal.



Case 547, April 23, 1907. A heifer in an advanced stage of loco poisoning.



Case 547, August 22, 1907. Completely recovered under treatment. Heifer was sold for fat beef.



Case 551, June 14, 1907. A horse in advanced stages of loco poisoning; weight 510 pounds.



Case 551, September 20, 1907. After course of treatment; weight 825 pounds.



Case 71, June 10, 1907. A locoed sheep, emaciated and extremely nervous.



Case 71, September 22, 1907. Recovered as a result of course of treatment.

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animal, a chronic loco, and its death was due not only to the effect of the loco poisoning, but doubtless to old age. The history of two of the animals treated is of special interest and is given below.

No. 551, belonging to Mr. Mosher, of Hugo, was received May 21. It had been locoed the previous season and had been fed on grain all through the winter. Plate XI, figure 3, shows its general appearance on June 14. It was a miserable animal—was very poor, its winter coat was still on in spots, and gave it an especially woe-begone appearance. It stood about in a listless way, with its head down, but when disturbed, as by a sudden noise, it would jump and rear up, showing the unstrung condition of its nervous system. It was 4 years old and weighed 525 pounds. The case seemed almost hopeless and hardly worth experimenting with. It was put on daily doses of Fowler's solu-

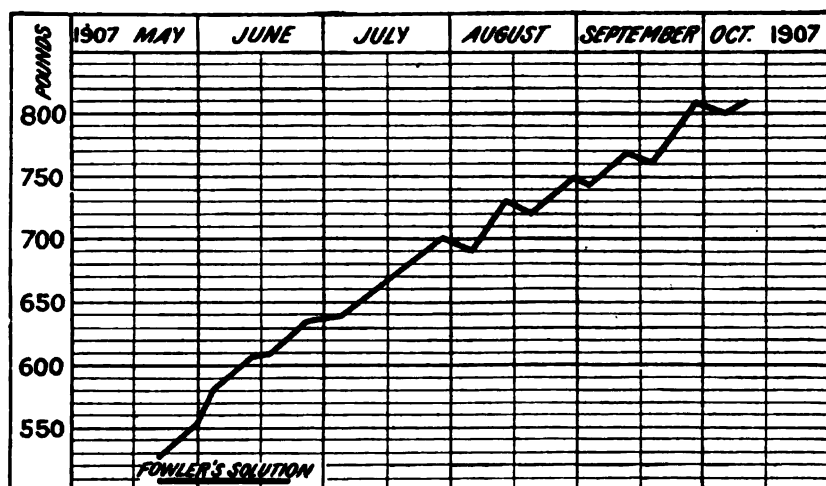


FIG. 29.—Curve of weight of horse No. 551.

tion and given some chop and oil meal daily in addition to its pasture feed. It gained rapidly. Figure 4, Plate XI, shows its appearance on September 20. The treatment was continued thirty-three days, but the animal was kept in the pasture through most of the season. In the fall it weighed 810 pounds and was a fine, sleek-looking horse. The curve (fig. 29) shows its increase in weight as the result of treatment.

No. 564 was a gelding belonging to Mr. Northrup, of Hugo. He was 7 years old, had been used as a work horse, and had been considered a gentle and valuable animal. Within two weeks preceding the time that he was brought to the station he had developed peculiar nervous symptoms—would throw himself, kick, and behave in a very erratic manner generally, so that it was considered unsafe to use him longer as a work horse. He was put on daily doses of Fowler's solu-

tion and fed a little chop each day. The treatment was continued for twenty-eight days, when he was taken by the owner and again put to work. He gave no further trouble and was later sold as a sound horse.

Of the others, No. 572 was treated for so short a time that it was impossible to say whether very much benefit was received from the treatment. It was a horse belonging to Mr. Dayton, was at the ranch only ten days, and it was difficult to say when it was discharged whether it had really made very much gain or not.

One horse was treated at Woodland Park. This animal was confined in a corral and fed chiefly upon old hay. Fowler's solution was administered in its drinking water. The horse was not under constant observation, as were the animals in Hugo, but it made steady gain under the treatment.

TREATMENT OF SHEEP IN 1907.

One sheep was treated in 1907. This was brought by Mr. Frank Hamp, and while the history of its feeding previous to the time when it was brought to the station was not known, it was pronounced by Mr. Hamp, an experienced sheepman, as a good case of loco poisoning. It was received June 10. It was in fair flesh, but in a highly nervous state. Its head was held high and shook with a peculiar vertical motion so that its teeth rattled together. It certainly deserved the name of crazy. It was put on one-twentieth grain doses of strychnin and showed marked improvement in less than two weeks. The treatment was continued for thirty-three days, when the nervous symptoms had disappeared, and it appeared all right in every way. This animal seemed to be a perfect cure. Plate XI, figures 5 and 6, show its condition when received, June 10, and when it was considered a cure, September 22.

A number of sheep that were left at the station by Mr. McIntyre and were considered locoes were kept during the season, but it was decided that none of them were cases of loco. They showed no tendency to eat the plant, would eat grass simply, and it was decided that the poor condition of the animals when received was largely the result of the presence of animal parasites. Some of them were treated for *Estrus ovis*, and all came out in the fall in good condition without any further treatment.

PART III.—RESULTS AND CONCLUSIONS.

CAUSE OF THE DISEASE.

The first and most important question arising was to decide whether the disease was caused by the loco plants. While the results of previous investigation cast much doubt on their poisonous character, the great majority of the stockmen were agreed that the loco plants were at the bottom of the trouble. Great interest, therefore, was attached to the feeding experiments. At first it appeared that it would be shown that they were harmless. The animals placed in the loco pasture not only did not suffer from any harmful effects of the weed, but, on the other hand, grew sleek and fat. The horses fed in the corrals showed no unusual symptoms. This went on for some weeks. When the effects of the poison began to show themselves they came very suddenly, and within a short time the animals succumbed entirely. The conditions in the feeding experiments were such that the deaths of these animals must have been caused by loco. As compared with animals under conditions in other respects identical, the loco eaters invariably grew poor and eventually died. In the cases where loco was fed exclusively it may of course be said that the trouble was a lack of nutrition in the plant. This possibly might have been the case in some of the horses of the first season, but in the other cases an abundance of other feed was provided and in spite of that the animals went down.

During the second season a careful record of weights was kept and curves made to show the changes during the season. The curves show in a striking way the fact that loss of weight always followed the feeding of loco. All the horses, cattle, and sheep that ate loco in any amount were affected. No. 10 was especially interesting, because, although he ate loco, he did it in moderation, so that he lived in a comparatively fair condition, but with less than the normal increment in weight.

During the third season, too, the curves of weight showed some loss whenever any considerable amount of loco was eaten. In the summer of 1907 there was no forced feeding of loco, but the animals experimented upon were kept constantly where loco was abundant, and a daily record was kept of what was eaten by the animals when they were in the field. It was noticed that the weights varied much, according to the amount of loco eaten. No. 10 kept on in the

third summer as he had before, with the same general result, being a rather poor specimen of a steer, but not suffering largely from his bad habit.

Of the previously suggested causes of the disease other than the plants themselves, most had no reasonable basis and no discussion of them is necessary. The theory of animal parasites, however, did have a definite experimental basis. It was noticed in the beginning of our work on horses and cattle that not only was there no unusual number of parasites present in the subjects, but in many cases the number was unexpectedly small. The horses had bots, as do nearly all horses, but the number of intestinal worms was remarkably small. In many of the cattle no parasites at all were discovered. Examination of the blood gave no evidence of blood parasites.

With the sheep, however, it was a different matter. The general presence of *Æstrus ovis* and *Thysanosoma actinioides* was very noticeable. Inasmuch as the symptoms produced by *Æstrus ovis* sometimes resemble closely those of loco, a skeptical person might well question the fact of loco poisoning. Sheep, too, are erratic about their feeding, and in many cases it is a matter of some difficulty to get exact evidence of any considerable feeding upon the weed. If we not had our experience with horses and cattle before experimenting with sheep, it is very likely that we should have considered Marshall's conclusions as confirmed by our observations. But having already acquired positive evidence in regard to other animals, we were not as likely to be skeptical in regard to sheep. After some experience we could, by post-mortem examination, distinguish locoes readily, but it is very difficult before death to tell a locoed sheep from one suffering from grub in the head.

SYMPTOMS OF LOCO POISONING.

The field work has added very little new material to what had already been published in regard to the symptoms of loco disease. It appears that in some cases authors had exaggerated these symptoms and many of the recorded symptoms can not be verified, but in general they were correct.

During the early period of loco feeding there are no symptoms of poisoning. Horses and cattle will eat quite freely of the weed for a considerable period with no apparent ill effects and may even gain considerably in flesh.

The first symptoms of loco poisoning in horses are in the gait. The animal walks with a peculiarly stiff motion. It staggers as it walks, frequently moving the hind limbs with a noticeable drag, as though they were partially paralyzed. Its hind legs may be kept

far apart, as if in an attempt to support itself. In stepping over an obstacle it lifts its feet unusually high with a jerking motion, and may leap unnecessarily high, as though unable to judge of distance. It may stumble and fall, getting up with a good deal of difficulty. The animal develops a solitary habit, staying away from the rest of the bunch. This solitary habit is supposed by some to be due to its desire to hunt loco, but is probably simply because of its general nervous depression. These symptoms may appear when the animal is in fairly good flesh. It becomes slow and "dopy." It may stand for hours without eating and not go to water for days. When it attempts to drink it may have difficulty in getting its head to the water, and, when it reaches it, may move its mouth as though attempting to eat rather than with the motions of drinking. This peculiarity in drinking is accompanied by equally peculiar motions in eating and may be very pronounced before the animal deteriorates much in flesh, as shown in Plate V, figure 1.

The animal may eat little but loco, going from plant to plant hunting the weed. More commonly it eats considerable grass with the weed, but as the disease progresses it eats less and less of anything.

Sometimes a horse in the early stages of the disease will be in constant motion, walking about in a restless and aimless sort of way. If such a horse is confined in a corral, it may walk around the corral constantly, apparently never resting. As the effect of the loco increases, the animal loses flesh, its coat becomes rough, and its eyes staring. A horse in this condition will stand hour after hour the very picture of dejection, showing no interest in anything. If approached suddenly it will rear, its legs flying about in a peculiarly purposeless way, and perhaps fall over backward. This is well shown in Plate V, figures 2, 3, and 4.

A locoed horse when driven may shy violently at some little thing, and it is dangerous to use such an animal. A horse that becomes locoed after being broken may become entirely unmanageable. It is impossible to lead it, back it, or tie it. Generally speaking, however, the crazy symptoms are not so noticeable as would be supposed from the popular accounts. Nearly all locoed animals, when much affected, are constipated.

Cattle show the same symptoms, but none in so marked a way. A steer will not rear, but a sudden noise will send a tremor through its whole body, and in walking it shows the same lack of muscular coordination that is seen in the horse. From the fact that one can approach close to one of these animals without being noticed, and that then the animals will suddenly rear and jump, it is popularly thought that the eyesight is affected. It does not appear, however, that there is really any defective vision, but that the apparent trouble with the

eyes is simply due to the general disorganized condition of the nervous system. Probably the trouble is mental rather than due to any difficulty with the eyes, although this is a difficult matter to decide positively. It is sometimes difficult to lead a locoed animal into a barn, as it is apparently afraid that the door is not high enough, or it may crouch as if to avoid something that is not present at all. This is well shown in Plate VII, figures 1 and 2, where the animals in going through a gate crouch and avoid an imaginary wire or an upper bar.

Commonly, a locoed animal is dull, but under especially exciting causes it may become almost frantic. This is particularly noticeable in steers taken off the range, which will sometimes go into paroxysms of rage and attack everything in sight. It is almost impossible to drive a locoed animal. It may run into the horse of the person driving it. When started in one direction it is sometimes almost impossible to turn it. Sometimes an animal after being started will go straight ahead at a uniform gait until it strikes some obstacle. If this obstacle happens to be a wire fence it will go through it, paying no attention to incidental cuts. This would not be so surprising in the case of a steer, for cattle under normal conditions will frequently go through a fence with little harm, but most horses are afraid of barbed wire, and even a small cut will stop them. Not so with locoed horses; they will sometimes rub along a fence, mangling themselves, but apparently suffering no pain. The condition of such a horse is shown in Plate VIII, figure 6. It is evident that the nerves of sensation are partially paralyzed. It is noticeable, too, that a horse that has been cured of loco has permanently lost much of its sensitiveness. The whip and spur sometimes make very little impression, although the animal may have been very sensitive before suffering from the loco poison.

In general, the symptoms in horses and cattle are very much the same. The lack of muscular coordination is more noticeable in horses, as would be expected from their more delicate nervous organization. The head of a locoed steer or cow will shake like the hand of a palsied man. Frequently in cattle there will be local accumulations of serum, especially under the jaw. These are not so likely to occur in horses. Abortion is a common phenomenon in locoed cows. By many stockmen this loss of the calf crop is sometimes considered one of the most serious of the effects of loco. As the disease progresses, the animals eat less and less and finally die of starvation.

Another symptom which was noticed in some of the cattle was a tetanic condition of some of the flexor muscles of the hind limb, so that the fetlock joint was flexed and the animal appeared to be "standing on its toes." This is shown in Plate X, figure 5.

The symptoms in sheep are similar to those in horses and cattle, but, as would be expected from the character of the animals, they are less noticeable. The locoed sheep walks in a stiff way, staggers, and becomes weak. It lies down frequently and does not keep up with the band. Its head may shake, although this is not a very common phenomenon. The herders say that locoed sheep are "foolish" or "crazy," and these terms describe very well the general appearance of the animals. Lambs are peculiarly susceptible to the influence of the poison and may succumb to it within two or three weeks, dying with very little loss of flesh.

It will be remembered that in the list of symptoms both high and low temperatures were mentioned. The results of the field work would seem to indicate that both classes of observers were right. In some locoed animals temperatures as high as 106° or 108° F. have been observed, and in a few cases subnormal temperatures. In a great majority of chronic loco cases, however, the temperature does not differ much from the normal, and it can be safely said that chronic loco poisoning does not have any distinct effect upon the body temperature.

PATHOLOGICAL LESIONS.

Loco victims always show marked anemia. This is indicated not only by the emaciation and paleness of the flesh, but by the excess of serous fluids of the body and by the deposits of organized serum in various parts of the body. This is more especially marked at the base of the ventricle of the heart.

EXAMINATION OF BLOOD.

Inasmuch as all locoed animals show marked symptoms of general anemia, a technical study of the blood is of considerable interest. The multiplicity of general duties at the station made it impossible to make any very large number of blood determinations. Some counts, however, were made in the second and third summers and the results are significant. Little seems to have been published in regard to the blood counts of cattle, and it would have been interesting if we could have made a large number of counts of both normal and locoed animals. The counts in the summer of 1906 were made by Scientific Assistant Klugh, and his work was checked off by counts made by two other assistants. In 1907 the blood counts were made by Field Assistant Clawson, and this work was checked by counts made by two others.

The normal number of red corpuscles for cattle at our station seemed to be something over 8,000,000. This is higher than recorded normals, but our station was at an altitude of nearly 5,000 feet, and

we would expect higher counts at this elevation. The locoed animals examined in 1906 averaged 5,138,333; those examined in 1907, 21 in number, averaged 7,250,238. This apparently shows a wide discrepancy. It is largely explained, however, by the fact that those examined in 1906 were nearly all in the last stages of the disease, while most of those examined in 1907 were undergoing treatment and in some cases had already made marked gains. These results are such as would be expected, emphasizing the fact of the marked anemia. The following table is significant as indicating the effect of treatment upon locoes, as it shows in general gradual improvement as the course of treatment progressed.

Number of red corpuscles in blood of number of normal and locoed cattle, the latter in various stages of treatment.

No. of animal.	Treated less than 1 week.	Treated 1 to 4 weeks.	Treated 4 weeks and over.	Not locoed.	Remarks.
33.....	6,180,000				} Died, bad jaw. Bad case.
36.....		7,580,000			
38.....		6,000,000			
43.....				8,000,000	
546.....			6,650,000		
546.....			9,600,000		
546.....			8,840,000		
554.....		9,917,000			
556.....		5,380,000			
556.....		8,900,000			
560.....	5,930,000				} Died.
42.....		6,979,000			
361.....	7,508,000				
557.....			8,520,000		
558.....		6,120,000			
546.....	6,810,000				
569.....	6,070,000				
557.....			8,220,000		
35.....			6,473,000		
Average.....	6,511,600	7,268,000	8,050,000	8,000,000	

An average of 12 locoes in 1907 showed 3,735 white corpuscles.

Tests of hemoglobin were made by the Talquist scale. Healthy animals varied from 85 to 98, the average being between 90 and 95. In the locoes examined in 1906 the hemoglobin was 70, while in 1907 it averaged 85. The lowest observed in 1907 was 75 and the highest 85. It should be borne in mind, however, that many of the 1907 locoes were on the road to recovery, and in some cases were in very good condition.

On July 21, 1906, 10 healthy sheep were taken at random from a herd that were in the shearing sheds, and an examination of hemoglobin made by the Talquist method. The average of these 10 gave 87.7. On the same date an examination was made of 14 locoed sheep, giving an average of 78.

WALLS OF STOMACH.

In nearly all locoes there is a diseased condition of the stomach. In acute cases the walls are very much inflamed. In chronic cases ulcers are commonly present. The ulcers are not so common in the stomachs of horses, but are almost invariably present in the fourth stomachs of cattle. In sheep one is apt to find inflamed walls rather than ulcers.

In these ulcers a microscopic examination shows that the mucous membrane is entirely destroyed. Sometimes other parts of the alimentary canal may be inflamed, or have small ulcers, but this is not a usual condition.

HEMOLYMPH GLANDS.

The hemolymph glands are apparently much more prominent in locoes than in normal animals. This is especially noticeable in cattle. In these animals the hemolymph glands seem to be very numerous in the thoracic cavities, in the connective tissue about the heart, and in that in front of the thoracic aorta. They are only less noticeable in the lumbar region of the abdominal cavity. In the literature on the hemolymph glands there seems to be nothing in regard to the question as to whether numbers of these glands have any pathological significance. Most of the investigations have been directed to physiological questions. There has been no opportunity for the author to make the extended observations which would be necessary to throw light on this question. Preliminary observations on a considerable number of animals in slaughterhouses make it seem probable, however, that the normal number is much smaller than in the case of locoes, and it seems possible that the condition of chronic anemia in these poisoned animals may be correlated with the number of hemolymph glands.

NERVOUS SYSTEM.

The central nervous system is generally in a hyperemic or congested condition. In a few cases clots were found in the lateral ventricles of the brain. We have never, however, found clots in the fourth ventricle, although this has repeatedly been said to have been the case by the earlier observers of loco phenomena.

The serous exudate in the epidural space is especially abundant and is more or less organized. Commonly it is particularly abundant about the points of exit of the spinal nerves. This condition is rarely absent in chronic locoes. It seems probable that the pressure of this

coagulated serum may be the cause of many of the nervous symptoms which are pronounced in loco victims. In some cases this coagulated serum is especially abundant in the lumbar region, and it might well be the cause of the partial paralysis of the hind limbs, which is frequently very marked.

In locoed cows and mares, generally speaking, the ovaries are found to be more or less diseased. They are generally small and hard, and frequently contain serous cysts.^a

In a number of cases it was found that the kidneys were somewhat diseased. This was not, however, the condition of the majority of the cases, and would not seem to be typical of loco poisoning.

VIRULENCE OF LOCO POISON.

TIME REQUIRED TO PRODUCE POISONING.

Previous authors vary greatly in regard to the length of time taken for loco to affect animals. Some say that it takes only a few days; others that the poison may be several years in producing marked effects. Our work seems to show that small amounts of the weed produce no appreciable effect, and that the poison is cumulative, so that the apparent results come quite suddenly after a period when the animal does not appear to be affected at all. The time from the beginning of the feeding to death in the animals experimented upon varied from two months and eight days to six months and nine days. It might be expected that there would be much variation in the time, because it is impossible to secure uniformity in conditions. There is a great difference in the individual susceptibility to the poison, and when animals are in the pasture some eat a very much larger amount of the loco than do others. When fed in the corral, too, on a mixture of loco and other feed, there will be an individual difference in the relative amount of loco eaten. Of the 1905 cattle dying of loco, the average length of life after the feeding was begun was five months and six days. Of these, No. 12, which was fed partly in the corral, died in three months and thirteen days. Of the other four, two died in six months and five days, and one in six months and four days. This would seem to indicate that the corral-fed animals succumbed the more quickly. In this, as in other cases, we must bear in mind, however, that while doubtless the corral-fed animals ate more loco, they may also have suffered somewhat from confinement and from an actual lack of proper food. This doubtless was true in the case of

^a Dr. John R. Mohler, chief of the Pathological Division of the Bureau of Animal Industry, calls attention to the fact that a cystic condition of the ovaries is a common condition in the horses and cattle of the West, and that this condition may not be due to the specific effect of the loco poison.

some of the animals of the first year. Then, too, generally speaking, when animals were first taken into the corral they were starved to give them an appetite for loco. In other cases some were fed loco exclusively for many days. This treatment doubtless would occasion considerable losses.

Of the 1905 horses the average length of life after commencing to eat loco was three months and seventeen days; those eating *Aragallus lamberti* lived three months and nineteen days; those eating *Astragalus mollissimus* lived two months and ten days.

In 1906 cattle lived on the average three months and seventeen days; the corral-fed lived three months and four days; the pasture-fed lived three months and seventeen days. The horses lived four months and nineteen days; the horses eating *Aragallus lamberti* lived four months and twenty-four days; those eating *Astragalus mollissimus* lived three months and fourteen days. Those in the *Aragallus lamberti* pasture lived four months and twenty-eight days; those fed on *Aragallus lamberti* in corral lived four months and twelve days.

It will thus be seen that in the first year the horses died sooner than the cattle, while in the second year the cattle lived the shorter time. This difference in results I will not attempt to explain fully. It may be said, however, that in the season of 1905 more of the horses than of the cattle were fed in the corral, and doubtless they suffered more from shortness of feed, especially as some of them were for a time fed on loco exclusively. In the season of 1906 one of the cattle had eaten loco during the latter part of the preceding season, but this time was not taken into account in the tabulation of the averages. This reduced the average of the cattle somewhat. Then there is reason to think that some of the second season's cattle were peculiarly susceptible to the poison. The cattle during the second season ate much more freely of the weed than did the horses. It must be recognized that the number of our cases is not large enough so that we can place implicit reliance on averages. All we can say is that the results are significant, but must be interpreted very carefully.

The animals eating *Astragalus mollissimus* died more quickly than those eating *Aragallus lamberti*. Of the horses of 1905, those eating *Aragallus lamberti* died in three months and nineteen days; those eating *Astragalus mollissimus* in two months and ten days. Of the horses of 1906, the one that was fed *Astragalus mollissimus* died in three months and fourteen days, while those eating *Aragallus lamberti* died in four months and twenty-four days.

The work upon sheep was of such a character that no general conclusions can be drawn as to the length of time for the poison to take effect.

Horses and cattle feeding freely and continuously upon *Aragallus lamberti* may live from three to six months. Horses feeding freely and continuously upon *Astragalus mollissimus* are not likely to survive longer than from two to four months. The fatal issue of the habit may be put off almost indefinitely, however, in the case of the moderate eater. It may live a year or several years, but it greatly deteriorates.

During the third season none of the animals were fed in the corral, but all were kept in a pasture where they had an abundance of grass, and they were not forced upon the loco except that they were held a part of the time where loco was abundant. As soon as they showed evident symptoms of being poisoned they were taken up for treatment instead of being permitted to go through the whole course of the loco disease, as in preceding seasons. An attempt was made to preserve life as long as possible and to produce cures when practicable. The natural result of these conditions was that the animals yielded to the poison much more slowly than in the preceding season. Another fact had, doubtless, some influence, namely, that the animals were in most cases the survivors of preceding experiments and were originally more or less immune to the effects of the loco.

AMOUNT OF LOCO NECESSARY TO PRODUCE POISONING.

The limitations of the field work and the comparatively small number of animals experimented upon make it impossible to state with any definiteness how much loco an animal must eat before showing symptoms of the poison. In all cases some pasture feeding was given to the animals kept in the corrals in order to give them suitably healthful conditions of life. When in the pasture, some would eat loco in considerable abundance, while others would devote themselves wholly or in a very large part to grass. It was our custom to put these animals into pasture at least one day in the week. It is to be presumed, too, that the amount of hay eaten while they were in the corrals would make some difference in the effect of the poison, and thus bring in wide individual variations.

Because of the small number of animals it was possible to subject only a few to a single line of treatment. In most cases each animal was a subject of several phases of the experiment. The amount of loco fed in the corrals, however, was weighed and a record kept for each animal. From these records we can make a rough estimate of the amount eaten before the symptoms of loco became evident. From these records it appears that about 400 pounds of *Aragallus lamberti* will loco the average horse or steer.

RELATIVE IMPORTANCE OF *ASTRAGALUS MOLLISSIMUS* AND
ARAGALLUS LAMBERTI.

The tentative conclusions reached at the end of the first year in regard to the effects of *Aragallus lamberti* and *Astragalus mollissimus* were amply confirmed by the work of the second and third years. *Astragalus mollissimus* is harmful only to horses. The loss of cattle from this plant is inconsiderable. This is not because the cattle can not be affected by the poison, but because they do not eat the weed. It was found in the corral experiments almost impossible to make cattle eat this species even when they were starved to it. Generally they would starve to death rather than take to it. So far as our experiments went, it may be said that cattle never eat *Astragalus mollissimus*. In regions where this weed prevails, and not *Aragallus lamberti*, locoed cattle are almost unknown. Examples of this are the regions about Holyoke, Colo., and Imperial, Nebr. The same thing is reported in the *Astragalus mollissimus* region in the Panhandle of Texas. About Santa Rosa, N. Mex., where the damage is commonly ascribed to *Astragalus mollissimus*, it is true, the author was told that possibly 10 per cent of the cattle and 50 per cent of the horses were lost annually. It would appear that perhaps *Astragalus mollissimus* was responsible for the loss of the cattle in this locality. The locoes of this neighborhood, however, have not been examined with any care, and it is possible that plants other than *Astragalus mollissimus* are responsible for the cattle loss. Our experience showed that not only would the cattle not eat *Astragalus mollissimus*, but that horses were not likely to take to it except because of scarcity of other food.

It is very different with *Aragallus lamberti*; not only horses, but also cattle and sheep, eat this readily. They are more likely to eat it during a scarcity of other food, but they will not only continue to eat it when other food is abundant, but may begin the habit at any time of the year. Of course they are more likely to start eating the loco in the winter and early spring and in the fall when the loco is green and succulent and the grass is dry. But even in midsummer, when supplied with abundant pasturage, if thrown in contact with loco plants they may eat considerable amounts—enough, even, to produce fatal results. Cattle eat *Aragallus lamberti* much more readily than do horses. Nearly all cattle will eat it more or less, and a large proportion will contract the habit. A great many horses, on the other hand, will not touch it even if run upon ranges covered with the weed. Consequently the loss of cattle from *Aragallus lamberti* is much greater than that of the horses.

Sheep, too, eat *Aragallus lamberti* very readily. It is always considered a misfortune to have a few sheep commence eating the weed,

for sheep are such imitative creatures that a few may lead a whole band astray. Some sheepmen immediately cut the throats of loco eaters on this account.

The symptoms of poisoning from *Aragallus lamberti* are like those from *Astragalus mollissimus*, but the effect does not come so quickly. The laboratory work conducted by Doctor Crawford has established the fact of the greater toxicity of *Astragalus mollissimus*. However, vastly the greater amount of damage is done by *Aragallus lamberti* because it is eaten so much more readily, and because it affects cattle and sheep as well as horses. Our pasture experiments all go to show that most horses when kept where *Aragallus lamberti* is abundant will sooner or later yield to the poison, and that the cattle succumb much more quickly to the attractions of the weed.

SUSCEPTIBILITY OF DIFFERENT BREEDS.

Sheepmen are unanimous in saying that the blackfaces are much more susceptible than the Merinos. This statement was abundantly confirmed by our observations.

In regard to cattle and horses, it is a matter of common observation among stockmen that the better bred animals are more likely to become locoed. They explained this in part, and probably reasonably, by the fact that animals accustomed to be taken care of do not know how to "rustle," and consequently take the food that comes first instead of hunting for better material. Reliable stockmen tell me that Durhams are more easily affected than Herefords. When our experiments started in Hugo, an old stockman remarked that two black steers in our herd would be the first to be affected, saying that this would be true not because of their color but because they were of better blood. The result showed the accuracy of his prophecy, for the black ones were the first to succumb. While we had a number of different breeds among our experimental animals, the numbers were not large enough for any general conclusions to be drawn. The experiments would indicate, however, that Herefords are decidedly less susceptible than Aberdeen-Angus cattle. The one steer of our herd that refused to eat any considerable amount of the loco was a scrub. In Colorado generally I saw more locoed Herefords than of any other breed, but this is doubtless explained by the fact that this is the most popular breed of range cattle.

AGE SUSCEPTIBILITY.

Marshall (1904) states that loco poisoning appears only in young sheep, generally speaking; never in those over 2 years old. Blankinship (1903) states that only young sheep and colts are affected. Older sheep and horses rarely acquire the habit. Our experiments

in Colorado show that while lambs suffer in an acute way from loco, the older sheep are by no means free from the disease.

In cattle and horses we had a general impression of the greater susceptibility of the younger ones, but this was hardly borne out by the facts. There seemed to be very little difference in the time taken to kill, whether the animals were young or old. We could determine but little about this in the first season's work, for most of the stock were young animals. During the second and third seasons, however, we had a number of old animals, especially among the horses, and there was no clear-cut difference in susceptibility to the poison because of difference of age. As is intimated elsewhere, there is a difference in individual susceptibility which must always be taken into account.

The apparently greater susceptibility of young animals, as noticed by other observers, may be due in part to the fact that there are more young animals than older ones, and hence apparently a greater number of cases among the young animals, and partly to the fact that many of the old animals are those that have survived because of an individual lack of susceptibility to the temptations of the plant.

EFFECT ON ANIMALS OTHER THAN HORSES, CATTLE, AND SHEEP.

We found it very difficult to get evidence of the effect of loco on mules. Some stockmen reported that they had seen locoed mules, while others said that mules were not affected. Considerable interest, therefore, attached to observations of the two mules which formed a part of our experiment in 1906. Unfortunately, both were old animals, and the poorest one died of old age. The other, however, was a fairly healthy animal and its death proved conclusively that mules could be locoed.

In the summer of 1907 a locoed mule was brought in to the station for treatment. It had the typical symptoms of loco poison. Its insensibility to barbed-wire fence cuts was especially marked, for it would run against a fence and rub itself along for two or three lengths, the wire cutting through the skin and into the muscle, but apparently occasioning no pain. When started up in the pasture it would trot along at a slow gait in almost a straight line, going for a long distance, sometimes until some obstruction in its way would stop it. This was an old animal, and while it would eat fairly well and we found it possible to dose it, it succumbed, probably from the combined influence of old age and the loco poison. There seems no longer any question in regard to the possibility of mules becoming locoed by eating *Aragallus lamberti*. It does not appear, however, that they are as liable to take to the plant as are horses.

In regard to other domestic animals and wild animals, the testimony is conflicting, and it is impossible now, with the evidence at hand, to pass on it. Some stockmen say that they have seen locoed antelopes. Others say that antelopes are not affected. Inasmuch, however, as there is no record of feeding experiments on antelopes, we must consider the matter as still an open question. Doctor Crawford's pharmacological work shows that rabbits are affected, and it must be presumed that other herbivorous animals may be susceptible to the poison of the weed. One reputable stockman told me of a man who fed cut loco to a lot of pigs, thinking it would be good for them, and locoed them all. The writer has even heard of locoed hens.

EFFECT ON MAN.

There are many stories of men poisoned from eating loco, but none of these can be authenticated. As a matter of fact, inasmuch as our work on horses, cattle, and sheep shows that the poison is very slow in taking effect and that an animal can eat the weed for quite a long time without any apparent harmful results, it seems unlikely that men have eaten enough to show the results of the poison. Pilgrim in 1898 investigated a case which was brought into the Hudson River State Hospital, and concluded that the cause was syphilis. The story by Janvier in Scribner's Magazine, Volume I, page 67, entitled "In Mexico," which is frequently quoted in this connection, was based on the properties not of the loco plant, but of stramonium.

LOSSES FROM LOCO POISONING.

Attempts have been made to get some estimate of the losses from loco poisoning, but it is impossible to make more than the rudest kind of conjecture. An intelligent horseman near Wray, Colo., estimated in 1905 a loss of 3 per cent of the horses. One man near Holyoke in 1906 lost from 12 to 20 per cent of the horses in one herd; another in the same locality in 1905 lost practically all his horses. The winter of 1906 was especially disastrous in New Mexico and Arizona. One man in the Estancia Valley in New Mexico lost 50 per cent of his horses. A conservative stockman near Santa Rosa, N. Mex., estimated that during one winter 50 per cent of the horses and 10 per cent of the cattle perished. Around Jerome Junction, Ariz., the estimated loss in the winter of 1906 was from 30 to 60 per cent, and correspondingly large losses were said to have taken place about Flagstaff, Ariz. Blankinship says that in some parts of Montana the loss of lambs reaches as high as 50 per cent. It has been estimated that the annual money loss in Colorado is between \$1,000,000 and \$2,000,000.

The death loss, however, may not be the largest factor in the account. The loss from deterioration of stock and from premature sales is very heavy. Many stockmen, when their herds commence to eat loco, sell out rather than run the risk of having a lot of locoed animals on their hands, and this loss means a very large reduction in their profits. This, of course, is a loss that it is impossible to make any estimate of. In many cases stockmen have been driven out of the business by the weed, and have even gone into bankruptcy. In some sections of Colorado, I am told, there are not more than one-fifth of the animals on the range that were found a few years ago, and this reduction of numbers is entirely on account of the loco. When it is remembered that these losses occur more or less widely through the semiarid region from the Canadian Provinces to Mexico, it will be seen that the total loss from these plants is extremely large.

DESTRUCTION OF LOCO WEEDS.

The question is naturally raised as to whether some means can not be devised of exterminating loco weeds from the range. The most obvious way of doing this is by cutting them out. This is entirely feasible if the land is worth the price of the work. There is an erroneous idea among many of the stockmen that plants will grow from any part of the root, and that cutting the plants is useless unless the whole root is dug up. While the plant is a perennial, the buds are all at the crown, and if this is cut off the plant is destroyed. This is brought out by Professor Blankinship very clearly in his paper of 1906. Actual experiments in Hugo in connection with our loco work there have proved the correctness of this view. Inasmuch as all the seeds do not germinate the first year, of course it is necessary to repeat the cutting in successive years, but that it is entirely possible to clear pastures of the loco there is no doubt.

Doubtless much can be accomplished by the use of a mowing machine at the time of flowering. The flower scapes of *Aragallus lamberti* are erect and a machine would cut out most of them and thus spoil the seed crop. The work of cutting out, however, is not as laborious as one might think, and it is very effective.

At the present time the most efficient way of combating the loco seems to be to cut it out. Where pastures are fenced in this can be done very easily. *Astragalus mollissimus* is much more easily cut out than *Aragallus lamberti*, as it grows in patches and rarely in any very large amount. On the open range, of course, this method of exterminating the loco is impracticable.

In many cases much can be accomplished by keeping animals away from ranges covered with loco during the time when feed is

short, inasmuch as they are much more likely to contract the habit when other food is lacking. Sometimes it may be profitable to feed for a short time, in order that the habit may not be formed.

The question was raised whether it might not be possible to kill out the loco plants by chemical means, that is, to use some substance that would kill the loco without injuring the grass. Although the prospect was not very hopeful, it seemed worth while to try the experiment. Plots of land were laid out with suitable control plots, and experiments were made by sprinkling with varying strengths of ammonium sulphate, copper sulphate, and iron sulphate. From the use of ammonium sulphate and copper sulphate there were no good results; if anything the loco seemed to grow more luxuriantly. It at first appeared that certain strengths of copperas were giving the desired results; the leaves of the loco were killed while the grasses were uninjured. The thing appeared so hopeful that an experiment on a larger scale was attempted, and a considerable patch of ground on the ranch of Mr. McIntyre, near Hugo, was treated in the fall and left over winter, and then treated again in the spring. It was thought that by this extended treatment it would be possible to determine whether the poisoning was effective or not. The result was, however, disappointing, and it was evident that these substances could not be relied upon as exterminators of the plant.

INSECTS DESTROYING LOCO PLANTS.^a

A large number of more or less destructive insects are found in the loco plants. Of this number the larvæ of *Walshia amorphella*, a small moth, and those of *Euxesta notata*, a minute fly, are especially active. They not only bore in the roots, but ascend into the leaf stems and flower scapes, and when very abundant kill large numbers of the plants. In 1905 it was noticed that an especially luxuriant loco pasture, where the *Astragalus mollissimus* grew in large bunches over a considerable area, was practically cleared of the plants in less than a month's time, and during the remainder of the season and through the summers of 1906 and 1907 very few plants were found there. All along the line of the southern branch of the Union Pacific Railroad in 1906 and 1907 there was a general destruction of *Astragalus mollissimus*. There were few large plants, most of those found being small, and in many cases they died early in the season. It is noticed, too, that these larvæ work so early in the season as very largely to kill the plants before the seeds are matured.

^a The insects mentioned under this heading have been identified by Dr. F. H. Chittenden, of the Bureau of Entomology of the United States Department of Agriculture, and a paper on the insects known to infect the loco weeds has been published as Bulletin 64, Part V, new series, of that Bureau (pages 33-42).

From conversations with observant stockmen it appears that *Astragalus mollissimus* comes and goes in cycles. Probably the insects increase to such an extent as to destroy the loco; then, because food is lacking, the insects largely die out, only to reappear again when the loco gets another good start.

The same larvæ live in *Aragallus lamberti*, but never seem to do so destructive work in this plant.

The question naturally arises whether these insects can not be encouraged and carried from place to place in order to exterminate the loco plants in other areas. In the summer of 1906 the question of colonizing the insects was raised in connection with our experimental work, but it was hardly deemed practicable. An examination of the more luxuriant loco pastures of western Nebraska showed that the insects were already there, and were likely to increase as rapidly as they would if we lent any additional aid. It appears probable from our observations in Colorado that the insects, at the present time, have put in their work so effectively that *Astragalus mollissimus* is likely to be not, perhaps, a rare plant, but very much reduced in numbers for possibly from two to five years.

The grub of *Cleonus quadrilineata* is especially destructive to *Aragallus lamberti*. A. B. Clawson, field assistant in 1907, spent considerable time in studying the work of this insect, and while his work is necessarily incomplete it is sufficient to indicate that this is one of the most important factors in keeping down the numbers of the *Aragallus lamberti* plants. The larva of this beetle when mature is very noticeable when one digs up the plants of *Aragallus lamberti* by the roots. It is a large white grub, and either lives in the roots or, in many cases, burrows in the outside of the root of the plants. It was found in abundance as early as the latter part of June. Perhaps more were found working upon the surface of the root, but in many cases they worked inside and destroyed a large part of the root of the older plants. It was noticed about the end of the first week in July that the pupæ were being found in the cases. These cases were apparently made largely of dirt and are commonly found on the side of the root, partly occupying spaces which had been eaten out by the grub. The cases are about 20 to 25 mm. long by perhaps half that in width, being irregularly oval in shape.

The first adult beetles were noticed coming from the pupal cases toward the end of July. At this time and during the first part of August the adults were quite fairly abundant. Just how much damage these grubs do to *Aragallus lamberti* it is difficult to say. Certainly they do not kill out the plants so thoroughly as those insects which infest *Astragalus mollissimus*. Their main work seems to be confined to the older plants, and, without any question, they hasten the death of these plants. In some pastures where the *Aragallus lamberti* had

grown into unusually large plants, it was noticed that they were all being destroyed. The chief work of the larva seems to be in limiting the number of years during which the plants may live.

It should be noted in this connection that the most destructive work of this insect comes after the period of flowering and seed formation, so that even when plants are killed they have already produced the annual crop of seed.

EFFECT OF DRY SEASONS.

It is a matter of common remark among stockmen that the loco weeds are most abundant in wet seasons. This is, doubtless, because the seeds have a water-resisting coat and do not germinate except under favorable conditions. Experimental attempts to raise loco weeds have shown this to be the case. Mr. V. K. Chesnut tells me that in Montana it was three years before they had a crop from sowing the seeds. Our own experiments gave similar results. It is not known how long the seeds will remain viable, but it is certainly several years.

During the past dry seasons in Colorado the loco plants have almost disappeared in some sections, this being probably due to the combined influence of insects and meteorological conditions. The insects have destroyed the old plants, and because of lack of favorable conditions for germination, new ones have not come in to take their place.

REMEDIES.

From the characteristics of the loco disease and its diagnosis, it is seen that it is the result of long-continued feeding. In most cases the amount of the plant eaten at any one time is small, much more being eaten of the grasses than of the loco. The effects of the poison in the more acute cases do not appear under about two weeks, and in most of the adults only after weeks and even months of feeding. Only very small doses of the poison are taken at any one time. When an animal is finally locoed it may be assumed that there is very little of the poison in its system in a form to be reached by an antidote. While doubtless an antidote for the poison may be found, it is not practicable to use the antidote as a remedy for chronic cases. In cases of acute poisoning an antidote can be used, but in the slow chronic cases it would be useless to attempt any such thing. It would seem possible, however, by the use of an antidote to prevent the original effects of the poison. Naturally the question arises as to whether it may not be possible to immunize the animals by small doses. From what has just been said it is evident that this is impossible in a poison of this character.

In the way of remedies, from a medical standpoint, there would seem to be three things to be attempted: First, to give something

that would destroy the appetite for loco; second, to give an antidote to counteract the effects of the poison; and, third, to give such remedies as would counteract the general effect of the poison and aid the animal in recovery. The chances of finding a substance that in one or a few doses would destroy the appetite for loco are not very great. Some attempts were made in this line, but, as perhaps might have been expected, were unsuccessful. Doctor Crawford's report on the finding of barium in the plants was made so late that no experimentation in regard to an antidote for this substance was possible, and this part of the work will have to be taken up later.

It seemed more hopeful to make the attempt to give remedies for the general condition of the animals with the hope of aiding them in building up against the effects of the poison. As has been indicated in the diagnosis, the chief effect of the poison is on the central nervous system, and this results not only in the marked nervous phenomena, but in anemia and general debility. In seeking a remedy, then, it seemed desirable not to attempt to use an antidote for the poison, but to use such remedies as would affect the nervous system and aid the animal in getting out of the anemic condition which is so pronounced. All the remedies tried had this end in view—to build up the patient with especial reference to the nervous system.

Some attempts at remedial measures were made in 1906. In the summer of 1907 this work was prosecuted more vigorously on the lines which were indicated by the results of the previous summer. Inasmuch as most chronic cases of loco are constipated, in all of the treatment we made sure that there was free action of the bowels. This was secured by occasional doses of Epsom salts, the condition of the animals being watched from day to day. During the summer of 1907 especial attention, too, was paid to the diet. All animals under treatment were fed a little grain daily. With this was mixed a little oil meal because of its laxative properties. When it was necessary to feed hay, alfalfa was used, largely because it also is laxative.

The remedies experimented with in 1906 were strychnin, potassium iodid, asafetida, caffein, arsenious acid, Fowler's solution, and valerian. As the result of this work we felt convinced that asafetida, valerian, caffein, and potassium iodid gave no good effects. In the use of strychnin the results were a little doubtful. Two of the cattle were cured during the strychnin treatment and there seemed no good reason why this drug should not have the credit of the cure, but it was true that other animals treated with strychnin died, in some cases partly at least as the result of the poison. None of the horses showed improvement from the use of strychnin. The fact that several of the animals showed indications of poisoning made it probable that strychnin was administered in too large doses, although only the minimum doses of the ordinary veterinary materia medica were used. It seemed probable that locoed animals were, perhaps, more

sensitive to the effects of strychnin and should be treated in especially small doses.

Arsenious acid effected a cure in one of the horses. This animal was treated at the Colorado Agricultural Experiment Station during the winter of 1906-7 under the general direction of Doctor Glover, and, while it was a clear-cut case of loco disease in the fall of 1906, with all the typical symptoms, it came out in the spring definitely and apparently permanently cured. This animal was used as a saddle horse during the summer of 1907 and at no time showed any of the signs of loco poison, if we except a slight loss of spirits.

One of the cattle, too, after it was treated with Fowler's solution was completely cured.

TREATMENT OF CATTLE IN 1907.

Fowler's solution.—Only one animal was treated with Fowler's solution in 1907. This steer was cured, but the cure was much slower than in some of the other remedies, although the subject was a favorable one.

Atoxyl.—One steer was treated with atoxyl and recovered. Two others did not do well and were afterwards put on strychnin and improved. The results from the use of atoxyl were not considered favorable.

Sodium cacodylate.—Five cattle were treated with sodium cacodylate. Of these 1 died, 1 improved, and 3 were cured. The one that died was an especially bad case and nearly hopeless from the start. The net result from the use of sodium cacodylate was very favorable.

Strychnin.—Fourteen head of locoed cattle were treated with strychnin. Two of these were of the experiment stock, while the others were brought in from ranches in the neighborhood for treatment. Of these 14, 2 showed improvement, 3 died, and 9 were cured. Of those that died one was very much improved so far as the loco poison was concerned, but died of pericarditis. The other two were very bad cases and one of them died by drowning, being too weak to get up after falling with its nose under water. One was treated with strychnin followed by sodium cacodylate, followed by another course of strychnin, being treated in all fifty-one days, and was discharged cured.

Strychnin and sodium cacodylate.—Two were treated with strychnin and sodium cacodylate at the same time and were cured.

TREATMENT OF HORSES IN 1907.

Seven horses and 1 mule were under treatment in 1907. In all these cases Fowler's solution was used, but one was also treated with strychnin and sodium cacodylate. No good results appeared from the use of the strychnin and sodium cacodylate. The mule died. It

was an old animal and a chronic loco, and the combination of troubles carried it off in spite of treatment. Old age without any doubt was an important factor in causing its death. Two of the others were not treated long enough for final results, but gave promise of recovery. Two showed distinct gain, but were not considered as positively cured. Two were distinctly cured.

GENERAL RESULTS OF TREATMENT.

RESULTS WITH CATTLE.

A careful study of the cases of cattle which were treated in the two summers leads to these conclusions:

1. Very advanced cases are generally hopeless; occasionally they may be cured, but ordinarily they will die.
2. Cattle may recover without any treatment if they are taken from loco and put upon good feed. There was one marked case of this kind in our herd in the summer of 1907.
3. Atoxyl is not effective as a remedy.
4. Fowler's solution is of doubtful efficacy.
5. Good results almost invariably follow the use of sodium cacodylate or strychnin, or the two used together. We felt the most certain of good results under the strychnin treatment. This opinion was reached partly as the result of a statistical study of the cases and partly as the result of the general impression made upon us in the course of treatment. Whenever, during the summer, we were particularly anxious that a given animal should recover, our tendency was always to put it upon the strychnin treatment.

RESULTS WITH HORSES.

The net result of the work of the two summers on horses shows:

1. As in cattle, advanced cases are hopeless.
2. As in cattle, recovery is possible in mild cases without treatment.
3. Strychnin does not give good results.
4. Fowler's solution can be used with the expectation of beneficial results.

PERMANENCY OF LOCO CURES.

It must not be supposed that an animal cured of loco will never eat the weed again. Probably in most cases if they have an opportunity they will go back to their old habits. When a horse has been cured it should be kept in a pasture free from loco. When a steer has been cured, it is probably best to put him upon the market as soon as he is in sufficiently good flesh rather than to run any risk of a second period of loco poisoning. It is rather interesting in this connection, however, that the two head of cattle which were cured in 1906 and were pastured on loco in 1907 continued in the loco pasture the whole

season, eating more or less of the weed, probably rather less, and showed little effect. It seems rather probable than in many of the cured cases they are not as likely to go back to eating loco as are animals that have never been subjected to its temptations before.

PREVENTION OF LOCO POISONING.

Not all animals will contract the loco habit, and those that do so will ordinarily commence the eating at a time when feed is short. It is a matter of common knowledge among stockmen that it is during the winter and spring seasons that animals are most likely to commence eating the loco weeds. This is especially noticeable in the spring, when the grass of the preceding season has been largely grazed off and the new grass has not started. At this time the loco weeds are, in ordinary seasons, green and attractive, and are frequently the most prominent plants in the plains vegetation. Thus many animals, forced by hunger, commence to eat the weeds, and while some of them abandon the loco after the grass starts, others contract the habit, which continues even after there is an abundance of good feed, and leads eventually to their death.

Much can be accomplished by the stockmen at this season in the way of prevention. In localities where the loco is largely confined to definite areas of the range it is possible to keep the animals away from the loco until after the grass has started, when there is likely to be very little trouble. In some cases feeding for a short period will bridge over the dangerous time, and while it may seem somewhat expensive to feed, it is evidently better than to have the stock die.

SUMMARY OF WORK ON REMEDIES.^a

1. Some locoed animals will recover if taken from the weed and fed well, without any treatment.
2. Most locoed animals will recover if they are taken from loco, fed carefully, and treated on the lines indicated by our experiments.
3. In all cases care should be taken to relieve constipation, either through the character of the food or by the use of magnesium sulphate.
4. Horses are best treated with Fowler's solution in daily doses of 15 c. c., continued for at least one month.
5. Cattle are best treated with daily doses of strychnin, the doses not exceeding three-twentieths of a grain, given hypodermically, and continued for one or two months. It is especially important that the dose should be small, as locoes are very susceptible to the bad effects from overdosing.

^a Further work on remedies during the summer season of 1908 confirmed the conclusions of the preceding years.

GENERAL SUMMARY.

For many years complaints have been made of the losses of cattle, horses, and stock in the semiarid region of the West from a disease which is popularly known as "loco," this name being given because of the crazy symptoms of the affected animals. The disease is prevalent from the Canadian provinces on the north to Mexico on the south, and the losses have been very great; so great, in fact, that in some sections stockmen have been thrown into bankruptcy, and sometimes most of the animals have been taken off the range. Some years ago the State of Colorado created a bounty for cut and dried loco, but such a large amount of it was collected and the bounty payments amounted to such large sums that the law was repealed for fear that the whole State would be thrown into bankruptcy.

Generally speaking, among the stockmen of this region the cause of the loco disease has been ascribed to certain leguminous plants known as the loco weeds, the chief of these being *Astragalus mollissimus* and *Aragallus lamberti*. During the last quarter of the nineteenth century this subject was investigated by a large number of people, but with very contradictory results. Attempts were made to isolate a poison from the loco plants, which succeeded apparently in the hands of certain authors, but the results of their work were not corroborated by others. Scientific men traveled largely through the loco region to observe conditions, and autopsies were made on a considerable number of animals. While it was evident that a disease of some kind was causing enormous losses through this semiarid country, the general result of the investigations of scientific men was to lead them to be very skeptical in regard to the supposed poisonous properties of the loco plants. It was noticed that most of the so-called locoed animals had been subjected to an unfavorable environment, especially in the way of feed, and the consensus of opinion among investigators seemed to be that some cause for the disease must be sought other than the loco plants. Some of the stockmen themselves were very skeptical in regard to the poisonous properties of the plants, and contended that with proper feed there would be no such thing as locoed animals.

Inasmuch as the losses were so heavy and the stockmen were anxious to have the subject investigated more thoroughly, the United States Department of Agriculture instituted an experiment in the summer of 1905 upon a larger and more thorough scale than had been

attempted before, hoping to prove or disprove the poisonous properties of the loco weeds. This experiment consisted in feeding cattle and horses upon pastures containing loco weed in comparison with others fed on pastures that were free from the loco weed, as well as feeding the loco plants in the corrals, and of making as extensive autopsies upon locoed animals as possible.

The experiments were intended (1) to prove whether the loco weeds would poison or not, (2) to demonstrate the symptoms of loco poisoning and the pathological lesions accompanying it, and (3) to determine whether or not remedial measures could be instituted for the relief of the loco-infested area.

The conclusions reached as the result of the work of three seasons are as follows:

1. There is no longer any question in regard to the poisonous properties of the loco plants. It was clearly demonstrated that animals eating these plants would succumb sooner or later to their poisonous action. This work, too, was abundantly corroborated by the pharmacological work of Doctor Crawford in the laboratory at Washington, by whom a poison was separated which was fatal to rabbits.

2. It was found that the symptoms of the loco disease were essentially like those which had been mentioned by the majority of stockmen. It was found that some of the symptoms were exaggerated, but, on the whole, the statements of the stockmen were amply corroborated. The more prominent symptoms are a staggering and uncertain gait, caused by a general disturbance of the nervous system, which leads in some cases to an apparent partial paralysis of the limbs and to a very distinct lack of muscular coordination. The animals eating loco eat more and more of it, although they do not in all cases acquire a passionate love for the weed, and sooner or later lose flesh and die of starvation.

3. In the post-mortem examinations it was found that there were certain quite definite lesions. The animals were strongly anemic. This anemia was indicated not only by paleness of flesh and actual loss of blood, but by serous deposits in various parts of the body. The blood was found to be poor in hemoglobin and commonly rather rich in leucocytes. A diseased condition of the stomach was a common accompaniment of the locoed condition, this being marked in cattle by ulcers in the fourth stomach. All the body fluids are rather unusually abundant, and this is particularly true of the fluid of the epidural space of the spinal canal, which is commonly more or less organized, so that the spinal canal frequently seems to be filled with a jelly-like substance. There is a hyperemic condition of the central nervous system, which in acute cases is accompanied by clots in the lateral ventricles. In females diseased ovaries are common.

4. The common loco plants in Colorado and adjacent territory are *Aragallus lamberti* and *Astragalus mollissimus*. Of these the more widely distributed is *Aragallus lamberti*, commonly known as the "rattleweed" or "white loco." The results of the experiment showed very clearly that *Astragalus mollissimus* was much the more virulent of the two species under consideration. This plant is more abundant in the southern part of the semiarid region, although not confined to that. In Colorado it is commonly found in the depressions and on adobe soil, while *Aragallus lamberti* is found upon the hillsides and upon sandy soil. *Aragallus lamberti* is not only common on the plains, but also extends in the mountains to an altitude of perhaps 8,000 feet.

5. Horses, cattle, and sheep are somewhat differently affected by these plants. In regions covered with *Astragalus mollissimus* the only common loosed animals are horses. Horses which eat this plant become poisoned ordinarily rather quickly and may die in a comparatively short time. Both cattle and horses eat *Aragallus lamberti*, but cattle, perhaps, rather more freely than horses, so that in regions where *Aragallus lamberti* is the more common loco plant the cattle are much more commonly affected than horses. In localities where *Astragalus mollissimus* is the only loco plant loosed cattle are very rare, indeed. Sheep eat both species, but for them also *Aragallus lamberti* is the more dangerous, inasmuch as they are more apt to eat this plant than *Astragalus mollissimus*.

6. It was found that there is a great difference in the individual susceptibility of animals to the loco poison, although most of them will succumb to the temptation and perish from its effects sooner or later. In regard to different breeds of animals there is a distinct difference, although the observations were not extensive enough for any broad generalizations. Generally speaking, the better-bred animals are more likely to be poisoned than those that have become accustomed to the country. Among sheep, black-faces yield much more quickly than Merinos. Among cattle, Durhams and Aberdeen-Angus were found to yield more quickly than Herefords.

7. In regard to remedial measures, the work of the experiment gives quite definite suggestions. It is clear that where land is sufficiently valuable to make it profitable to pay for that amount of labor, it is entirely feasible to cut out all of the loco weeds. This is particularly easy in regard to *Astragalus mollissimus*, because it grows in comparatively small patches. Where *Aragallus lamberti* is abundant the work would be more difficult, and in some cases the land is hardly worth the expense of the labor. It is evident that in the case of fenced pastures it frequently will be profitable to destroy the loco weeds in this way.

In the case of open range it is probably impracticable to arrange any means of doing this.

In many cases much can be accomplished by keeping animals away from ranges covered with loco during the time when feed is short, inasmuch as they are much more likely to contract the habit when other feed is lacking. Sometimes it may be profitable to feed for a short time, in order that the habit may not be formed.

It is absolutely necessary that animals which have acquired a taste for loco should be removed from temptation.

Except in very advanced cases, the treatment of locoed animals can be undertaken with every reason to expect good results. The first essential, of course, is to remove to a pasture which is free from loco. Their feed should be so regulated as to produce looseness of the bowels, or if this can not be done they can be dosed with some laxative, such as magnesium sulphate. Green alfalfa is one of the best feeds. If the animals are being fed on grain, it is well to mix oil meal with the grain. The necessity of producing a loose condition of the bowels can not be too strongly emphasized.

Some animals will recover under this treatment without recourse to medicine. With most animals, however, recovery is hastened by the administration of strychnin or sodium cacodylate, or both, in the case of cattle, and of Fowler's solution in the case of horses. All these medicines should be given in small doses and continued for a considerable period of time, seldom less than thirty days. The daily doses of strychnin should not exceed three-twentieths or four-twentieths of a grain (or 0.009 to 0.012 gram). Sodium cacodylate should be given in daily doses of 6.2 grains (or 0.4 gram). The strychnin and sodium cacodylate are best given by hypodermic injection. Fowler's solution should be given in doses of 15 to 20 c. c. in the grain or in the drinking water.

Recovery will in most cases be slow, as should be expected from the slow incipience of the disease; but care will bring the animal to a practical cure in the majority of cases. Cured animals do not seem as likely to eat the loco again, but it is best not to subject them to temptation.

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INDEX.

	Page.
Abortion produced by loco.....	21, 25, 61, 94
Age susceptibility.....	102
Antelopes poisoned by loco.....	24, 104
<i>Aragallus albiflorus</i> , synonym of <i>Aragallus lamberti</i>	38
<i>Aragallus lamberti</i> —	
Description, distribution.....	37
Experimental feeding.....	47-68
Less poisonous than <i>Astragalus mollissimus</i>	101
<i>Aragallus sericeus</i> , synonym of <i>Aragallus lamberti</i>	38
<i>Aragallus spicatus</i>	30, 38
<i>Aragallus splendens</i>	30
Arizona, loco poisoning in.....	40
Arsenic, treatment with, in 1906.....	78
<i>Astragalus arrectus</i>	32
<i>Astragalus bisulcatus</i>	25
<i>Astragalus caryocarpus</i>	17
<i>Astragalus crotolarie</i>	17, 18
<i>Astragalus dorycnoides</i>	28
<i>Astragalus haydeniensis</i>	25
<i>Astragalus hornii</i>	16, 17
<i>Astragalus lagopus</i>	28
<i>Astragalus lentiginosus</i>	16, 18
<i>Astragalus lentiginosus</i> var. <i>fremonti</i>	17
<i>Astragalus lotiflorus</i>	25
<i>Astragalus menziesii</i>	16
<i>Astragalus mollissimus</i> —	
Description.....	38
Distribution.....	39
Experimental feeding.....	45, 47, 48, 50, 51, 62
More poisonous than <i>Aragallus lamberti</i>	101
<i>Astragalus mortoni</i>	17
<i>Astragalus oocarpa</i>	17
<i>Astragalus palousensis</i>	28
<i>Astragalus purshii</i>	32
<i>Astragalus reventus</i>	32
<i>Astragalus spaldingii</i>	28, 32
<i>Astragalus spicatus</i>	28, 29
<i>Astragalus splendens</i>	28, 29
Atoxyl, experiments with.....	83
Bibliography.....	117
Bison, distribution of loco seeds by.....	40
Black-face sheep more easily affected than merinos.....	102

	Page.
Blood, examination of, in locoed animals.....	95
Breeds of animals, different, effect of loco on.....	102
Cattle—	
Experimental feeding of.....?	47, 56, 71
Experiments with remedies in 1907.....	83
Summary of treatment of, in 1907.....	110
Cause of loco disease.....	91
<i>Cleonus quadrilineata</i>	107
Conclusions derived from literature.....	34
Cycles, loco growth in.....	107
<i>Cysticercus tenuicollis</i>	31
Darling pea, poisonous to stock.....	41
Distribution of <i>Aragallus lamberti</i> and <i>Astragalus mollissimus</i>	37
Dry seasons, effect of, on loco.....	108
Durham cattle more easily affected than Herefords.....	102
Emmenagogue, loco as an.....	21
<i>Euzesta notata</i>	106
Experimental work, scope and details.....	42
Experiments at Woodland Park, Colo.....	71
Experiments with cattle—	
First season.....	47
Second and third seasons.....	56
At Woodland Park, Colo.....	71
Experiments with horses—	
First season.....	49
Second and third seasons.....	62
At Woodland Park, Colo.....	71
Experiments with sheep.....	66
Extermination of loco weeds.....	30, 105
Fertile soil, relation to loco plants.....	40
Fowler's solution, experiments with.....	83
Goat, locoed.....	53
Gompholobium, loco weed in Australia.....	24
<i>Hammonchus metastrongylus</i>	31
Hemolymph glands in locoed animals.....	97
Hereford cattle less affected than other breeds.....	102
Historical summary of loco disease.....	15
Horses—	
Experimental feeding of.....	43, 62, 71
Experiments with remedies in 1907.....	88
Summary of treatment of, in 1907.....	110
Imperial, Nebr., experiment at.....	73
Indigo—	
Disease.....	28
Plant.....	41
Insects destroying loco plants.....	106
Lambs peculiarly susceptible to loco poisoning.....	95
Lesions, pathological, of loco victims.....	95
<i>Leucocrinum montanum</i>	22
Literature of loco disease—	
Conclusions from.....	34
Summary and review.....	15
Loco cures, permanency of.....	111

	Page.
Loco, origin of name.....	15
Loco plants, list of.....	36
Loco poison—	
Amount necessary to poison.....	100
Effect on man.....	104
Prevention of.....	112
Susceptibility of different breeds of animals.....	102
Time before symptoms appear.....	98
Virulence.....	98
Loco weeds, destruction of.....	105
Losses from loco poisoning.....	104
<i>Malvastrum coccineum</i>	24
Man—	
Alleged poisoning by loco.....	25
Susceptibility to loco poisoning.....	104
Merino sheep less affected than black faces.....	102
Mule, experimental feeding of.....	64
Mules, susceptibility to loco poisoning.....	103
"Nenta disease" of South Africa.....	29, 41
Nervous system in locoed animals.....	97
New Mexico, loco poisoning in.....	40
Northwest Territories, loco in.....	28, 29, 31
<i>Oestrus ovis</i> in sheep, symptoms like loco.....	66, 68, 69, 70
Ovaries, diseased, in locoed animals.....	98
<i>Oxytropis lamberti</i>	16, 17, 18, 19, 21, 22, 23, 24, 25, 28, 29
Pastures used in experimental work, description of.....	44
Pathological lesions of locoed animals.....	95
Pea-eating disease.....	29
Permanency of loco cures.....	111
<i>Physostigma venenosum</i>	24
Plants known as loco plants.....	36
Prevention of loco poisoning.....	112
Problem of loco poisoning, statement of.....	42
Rattlesnake weed.....	28
Remedies—	
Discussion.....	73, 108
Experiments with cattle in 1907.....	83
General summary.....	112
Proposed in literature.....	32
Results in 1906.....	81
Results and conclusions from experiments.....	91
Review of literature.....	15
Sagebrush, poisoning by.....	41
<i>Sarcocystis tenella</i>	31
Sheep—	
Experiments and discussion.....	66, 69
Experiments with remedies in 1907.....	90
Sodium cacodylate, experiments with.....	83
Soil, relation of loco plants to.....	40
<i>Sophora sericea</i>	16
Spinal column, serous exudate.....	97
<i>Stipa viridula</i>	24

	Page.
Stomach, diseased condition of, in locoed animals.....	23, 97
Strychnin—	
Experiments with cattle in 1907.....	86
Treatment with, in 1906.....	75
Summary—	
General.....	113
Of first season's work.....	54
Susceptibility of different breeds of animals to loco poison.....	102
<i>Swainsona galegifolia</i>	41
Symptoms—	
Of loco poisoning.....	92
Mentioned in literature.....	32
Temperature of locoed animals.....	95
Time required to produce loco poisoning.....	98
<i>Thysanosoma actinioides</i>	31, 66
Treatment, general results.....	81, 111
Ulcers in stomach.....	23, 97
Virulence of loco poisoning.....	98
<i>Walshia amorphella</i>	106
Woodland Park, Colo., experiment at.....	71
<i>Zygadenus elegans</i>	22

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FILTRATION EXPERIMENTS WITH
BACILLUS CHOLERÆ SUI.

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BUREAU OF ANIMAL INDUSTRY,
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SIR: I have the honor to transmit herewith, and to recommend for publication as a bulletin of this Bureau, the accompanying manuscript entitled "Filtration Experiments with *Bacillus cholerae suis*," by Dr. C. N. McBryde, of the Biochemic Division.

The subject-matter of the paper, while technical in its character, has a direct bearing on the hog cholera problem. In a previous publication issued by this Bureau (Bulletin 72, The Etiology of Hog Cholera) it was first shown that the so-called hog cholera bacillus, *B. cholerae suis*, could not be regarded as the definite and sole cause of hog cholera, since the disease could readily be produced by an ultra-microscopic virus in the blood of sick hogs, which virus had repeatedly passed through a filter that effectually prevented the passage of *B. cholerae suis*.

This discovery naturally caused widespread discussion among scientific investigators upon this subject throughout the world, some of whom have questioned the accuracy of the findings published in the bulletin referred to. The experiments described in the present paper have been carried out to meet these criticisms and have resulted in confirming the correctness of the work previously published.

Respectfully,

A. D. MELVIN,
Chief of Bureau.

HON. JAMES WILSON,
Secretary of Agriculture.

CONTENTS.

	Page.
Introductory.....	5
Filters used.....	7
Arrangement of filters.....	7
Technique employed in filtrations.....	8
Cultures used.....	9
Experiments with Berkefeld filters.....	11
Summary of experiments with Berkefeld filters.....	13
Discussion of Berkefeld filtrations.....	13
Experiments with Pasteur-Chamberland filters.....	14
Summary of experiments with Pasteur-Chamberland filters.....	25
Discussion of Pasteur-Chamberland filtrations.....	26
Rate of filtration of Berkefeld and Pasteur-Chamberland filters.....	28
Granule formation in cultures of <i>B. cholerae suis</i>	29
Summary of results.....	30
Conclusions.....	31

ILLUSTRATION.

	Page.
FIG. 1. Apparatus for fractional filtration, designed for use with Pasteur-Chamberland or Berkefeld filters.....	8

FILTRATION EXPERIMENTS WITH *BACILLUS CHOLERÆ SUIIS*.

INTRODUCTORY.

In the early part of 1905 Dorset, Bolton, and the writer^a published a paper on the etiology of hog cholera, in which it was first definitely shown that hog cholera is due to a filterable virus and not to *Bacillus cholerae suis* as had been previously supposed. The conclusions reached in that paper were based on an extensive series of filtration experiments in which the blood serum of hogs sick of hog cholera was filtered through Berkefeld and Chamberland filters in order to exclude *B. cholerae suis*. In these experiments a large number of nonimmune, healthy hogs were injected with the filtered serum, which was carefully tested in every instance prior to injection for *B. cholerae suis*. The filtrates were proven to be free from that organism by either incubating the entire filtrate or else taking out large portions for subcultures, and also by the injection of large portions into guinea pigs and rabbits, animals which are extremely susceptible to *B. cholerae suis*. These experiments with the filtered serum of hogs sick of hog cholera and proven to be free from *B. cholerae suis* showed that if such serum was injected subcutaneously into well hogs it caused typical hog cholera with great regularity, whereas the same serum injected into small laboratory animals which are naturally very susceptible to *B. cholerae suis* was entirely without effect. In many of these experiments rabbits were injected with as much as 20 c. c. of the filtrates without any subsequent ill effects, whereas hogs injected with like amounts of the filtrates died of typical hog cholera.

These results, coupled with others which developed in the course of this investigation, led to the following conclusions: (1) That in the disease known as hog cholera there is some other etiological factor present besides *B. cholerae suis*; (2) that this other factor is an ultra-visible virus sufficiently small to pass through the pores of Berkefeld and Chamberland filters; (3) that this ultra-visible virus is the true cause of hog cholera, and that *B. cholerae suis*, when present in the blood and tissues of animals sick of hog cholera, is probably a secondary invader and at most an accessory factor in the disease.

These conclusions with regard to the etiology of hog cholera, being decidedly revolutionary in character, attracted widespread attention

^a Dorset, M., Bolton, B. M., and McBryde, C. N. The Etiology of Hog Cholera. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 72. Washington, 1905.

among scientific investigators in other countries, and it is gratifying to state that our results have been confirmed by many well-known workers in various parts of the world. Thus in Germany the same results were obtained by Ostertag and Stadie in the Hygienic Institute of the Veterinary High School at Berlin, and by Uhlenhuth and his coworkers, Hübener, Xylander, and Botz, in the laboratories of the Imperial Board of Health at Berlin. Similar results were obtained by Hutyra in Austria, by Stockman and McFadyean in England, by Theiler in South Africa, and within the past year by Carré, Leclainche, and Vallée in France. In our own country our results have been confirmed by McClintock, Boxmeyer, and Siffer. It is thus seen that our conclusions with regard to the etiology of hog cholera are not lacking in confirmatory proof.

Certain criticisms, however, have been made of our earlier work on the etiology of hog cholera, referred to above. In 1907 Lourens,^a subdirector of the State Serum Institute at Rotterdam, published an article on the filterability of *Bacillus suipestifer* in which he makes the statement that the so-called hog-cholera bacillus, known as *B. cholæræ suis* or *B. suipestifer*, is capable of passing through filters of the Chamberland and Berkefeld types, composed, respectively, of unglazed porcelain and infusorial earth. He claims that the ability of *B. cholæræ suis* to pass through filters of the types mentioned is due to a property which this bacillus possesses of breaking up into granules sufficiently small to pass through the pores of the filter. He also asserts that *B. cholæræ suis* is the cause of hog cholera and that none of the investigators who have conducted filtration experiments to show that hog cholera is due to a filterable virus have afforded sufficient and convincing proof that the filtrates which they employed did not contain *B. cholæræ suis*.

While we had no doubts as to the accuracy of our earlier filtration work, nor any doubt whatever that hog cholera is due to a filterable virus, especially since our earlier work has been confirmed by such well-known investigators as those cited above, we nevertheless felt that such criticisms should not be allowed to pass unnoticed, and it was with a view to meeting these criticisms that the experiments described in the present paper were undertaken.

In connection with our earlier work on the etiology of hog cholera the writer carried out a series of filtration experiments with cultures of *B. cholæræ suis*. This was done as a further test of the efficiency of the filters used—that is, to determine whether filters of the Berkefeld and Chamberland types could be relied upon absolutely to restrain or keep back *B. cholæræ suis*.

^a Lourens, Louis F. D. E. Untersuchungen über die Filtrierbarkeit der Schweinepest-bacillen (*Bac. suipestifer*). Centralblatt für Bakteriologie, abt. 1, orig., band 44, heft 5, pp. 420-427, Aug. 20; heft 6, pp. 504-512, Aug. 31; heft 7, pp. 630-648, Sept. 17. Jena, 1907.

Since the publication of Lourens's paper a second series of filtration experiments with bouillon cultures of *B. cholerae suis* has been carried out by the writer, and the results obtained, together with those obtained in the earlier experiments referred to above, none of which have hitherto been published, are now recorded in detail.

FILTERS USED.

The filters used were Berkefeld laboratory cylinders Nos. 5 and 7, and Pasteur-Chamberland bougies "F" and "B," and were of the same types as those used in the filtration experiments described in Bulletin 72.

A circular which accompanied the Pasteur-Chamberland bougies stated that these filters were manufactured in France and imported into this country, and this was also stated on the cardboard boxes in which the bougies were packed. The individual bougies were labeled in French as follows: "F [or "B"] Filtre Chamberland, Système Pasteur, H. B. & Cie., Choisy-le-Roi."

New filters were used for each experiment, except in the case of experiments 7 and 8 with the Pasteur-Chamberland filters, where filters were employed which had been used once and then cleansed according to the methods adopted by Lourens.

Before being used the filters were first tested by submerging them in water and then forcing compressed air through their walls. If the air came through the walls of the filter in the form of small bubbles or beads the filter was regarded as being free from flaws, but if large air bubbles formed on the sides this was taken as an indication of possible defects or flaws, and such filters were discarded. This method of testing the filters is similar to that employed by Lourens.

ARRANGEMENT OF FILTERS.

In the first three experiments with Berkefeld filters and the first three experiments with the Pasteur-Chamberland type the filters were attached to ordinary side-arm or suction flasks and the filtrates collected in one portion. In the remainder of the experiments the fractional method of filtration was used, for which a special form of apparatus was devised. The apparatus, which is shown in figure 1, may be used with either the Berkefeld or the Chamberland type of filter. It consists essentially of a double side-arm suction flask with which the filters are connected above by means of rubber stoppers and a protected outlet below connected with the lower end of the suction flask by a short piece of heavy black rubber tubing. The outlet consists of a glass tube surrounded by a glass shield which is open at the lower end and is plugged with cotton to protect the outlet tube from dust. The outlet is controlled by a pinchcock above.

One arm of the suction flask is connected by means of heavy rubber tubing with a vacuum gauge and suction pump. The other arm is connected by means of rubber tubing with a glass inlet tube plugged with cotton, the admission of air into the suction flask being controlled by means of a screw pinchcock. (See fig. 1.)

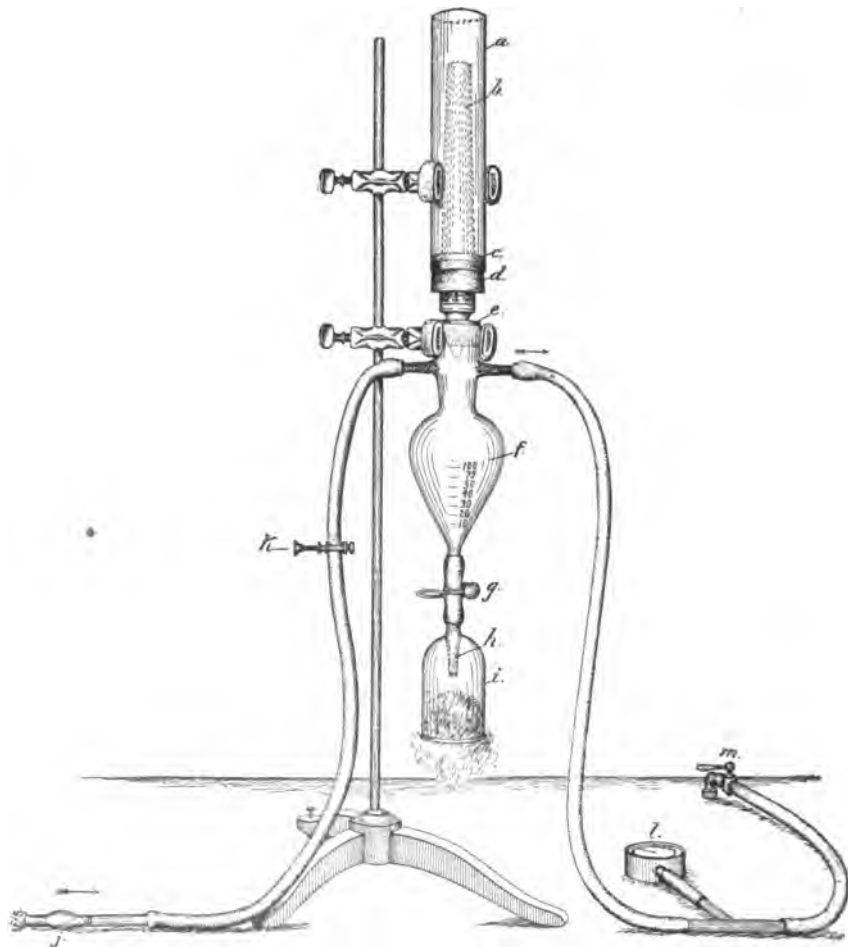


FIG. 1.—Apparatus for fractional filtration, designed for use with Pasteur-Chamberland or Berkefeld filters. *a*, Glass mantle surrounding filter; *b*, Chamberland filter; *c*, paraffin joint; *d* and *e*, rubber stoppers; *f*, double side-arm suction flask; *g*, pinchcock controlling outlet from suction flask; *h*, outlet tube surrounded by glass shield and attached to lower end of suction flask by means of short rubber tubing; *i*, glass shield fused to and surrounding outlet tube as a protection against contamination when the filtrates are drawn off; *j*, glass inlet tube plugged with cotton, for admitting air into suction flask; *k*, pinchcock governing the admission of air into flask; *l*, vacuum gauge; *m*, stopcock connected with vacuum pump.

TECHNIQUE EMPLOYED IN FILTRATIONS.

In conducting a filtration with the apparatus described, the pinchcocks *g* and *k* are first closed off and the vacuum applied at *m*. The filtration is then allowed to proceed until the filtrate reaches the

desired level in the suction flask *f*. As soon as the suction flask is exhausted of air the rubber tubing above the pinchcock *g* will be seen to collapse and will remain so as long as the vacuum is applied. When the filtrate reaches the desired level in the suction flask the vacuum is closed off and the air gradually admitted into the suction flask by means of the pinchcock *k* through the glass inlet tube plugged with cotton (*j*). The admission of air into the suction flask may be regulated by watching the rubber tubing above the pinchcock *g*, which expands as soon as the vacuum within the suction flask has been neutralized. The filtrate which has collected in the suction flask is now drawn off into a sterile Erlenmeyer flask by means of the pinchcock *g* through the glass outlet tube *h*. In withdrawing the filtrate an assistant removes the cotton plug from the glass shield surrounding the outlet tube, steadying the shield at the same time. The neck of the Erlenmeyer flask, which is to receive the filtrate, is then introduced within the shield beneath the outlet tube and the filtrate allowed to flow out by releasing the pinchcock above. The inlet tube is now closed off again by means of the pinchcock *k*, the vacuum reapplied at *m*, and another portion of the filtrate collected and drawn off as before. In this way as many fractions may be collected as are desired.

Dry heat is used for the sterilization of all glass portions of the apparatus except the glass mantle *a* surrounding the filter, which being outside of the filter does not require sterilization. Both arms of the suction flask should be plugged with cotton to prevent dust particles from being drawn into the flask. The filters and all rubber connections are sterilized by boiling in distilled water for from one-half hour to one hour. After the apparatus is set up all joints should be carefully paraffined.

CULTURES USED.

Five different strains of *Bacillus cholerae suis* were used. These cultures were obtained from different sources, either directly from hogs which died of hog cholera or from guinea pigs inoculated with cultures obtained from hogs which had died of hog cholera. The cultures were grown on the various laboratory media and their biological and morphological characters carefully noted. All of the cultures used were virulent for guinea pigs and rabbits.

A brief record of the different cultures is appended.

1. *Culture Gp 3998 S*.—This culture was obtained from guinea pig 3998, which died from the inoculation of a culture obtained from hog 1086. Hog 1086 was a nonimmune animal which served as a check in the Iowa experiments. The animal was exposed, together with certain other treated hogs, to a natural outbreak of hog cholera. As a result of the exposure the animal contracted the disease and died with typical cholera lesions, *B. cholerae suis* being obtained in pure

culture from the heart blood and spleen. A subculture obtained from agar plates made from the spleen of hog 1086 was used for the inoculation of guinea pig 3998. The guinea pig died within six days, and *B. cholerae suis* was recovered in pure culture from the spleen and heart blood. A subculture from agar plates made from the spleen of guinea pig 3998 was cultivated on the various laboratory media and found to correspond with *B. cholerae suis* in all respects. This culture, which we have designated Gp 3998 S, was used in the first four experiments with Berkefeld filters, and the first two experiments with Pasteur-Chamberland filters.

2. *Culture Gp 4692 S.*—Obtained from the spleen of guinea pig 4692, which died from the injection of 1 c. c. of unfiltered, undiluted blood serum of hog 1208, an experiment hog carrying the Scribner strain of disease in the experiments at Washington (see "Experiments with tail blood," Bulletin 72, page 38). This culture was used in a number of the culture experiments described in Bulletin 72 and was found to correspond with *B. cholerae suis* in all of its cultural characters.

3. *Culture H 2199 S.*—This culture was obtained directly from hog 2199, which served as a check in the Iowa immunity experiments. The animal died from the injection of 2 c. c. of unfiltered disease-producing blood from the Scribner and Syphax strains of disease. A description of the Scribner and Syphax outbreaks may be found in Bulletin 102^a of this Bureau. The autopsy on hog 2199 revealed typical cholera lesions and *B. cholerae suis* was obtained in pure culture from the spleen. This culture, designated as H 2199 S, was cultivated on the different laboratory media and was found to correspond in all of its cultural characters with *B. cholerae suis*.

4. *Culture H 2450 S.*—This was obtained directly from hog 2450, a western hog used in the Iowa experiments. Hog 2450 was an uninoculated check and was placed in the exposure pen (for description of exposure pen see Bulletin 102, page 12) along with certain treated hogs in order to test the virulence of the disease prevailing in the exposure pen at the time. The animal contracted hog cholera in the usual time and died with characteristic lesions of the disease. Cultures from the spleen were grown on the different laboratory media and found to correspond in all respects with *B. cholerae suis*.

5. *Culture H 2228 S.*—This culture was obtained directly from hog 2228, which died as a result of an injection of 5 c. c. of defibrinated blood obtained from an outbreak of hog cholera in Iowa. Hog 2228 exhibited characteristic cholera lesions at autopsy and agar plates from the spleen revealed *B. cholerae suis*. This culture, like all of the others, was cultivated on the various laboratory media and was found to correspond with *B. cholerae suis* in all of its cultural characters.

^a Dorset, M., McBryde, C. N., and Niles, W. B. Further Experiments Concerning the Production of Immunity from Hog Cholera. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 102. Washington, 1907.

EXPERIMENTS WITH BERKEFELD FILTERS.**EXPERIMENT 1.**

A flask containing 400 c. c. of neutral beef broth was inoculated with culture Gp 3998 S and placed in the incubator. At the end of twenty-four hours the flask was well clouded and 200 c. c. of the culture was then passed through a Berkefeld laboratory cylinder No. 5. The vacuum used was 20 inches and the time occupied in filtration was one and one-half hours. The filtrate was collected in one portion in an ordinary side-arm flask and placed at once in the incubator. At the end of eighteen hours the filtrate, which came through the filter perfectly clear, was distinctly clouded. Agar plates from the filtrate showed *B. cholerae suis* in pure culture. A subculture made from the agar plates was grown on the different laboratory media and found to correspond in all cultural characteristics with the original culture. The results obtained in this experiment show that *B. cholerae suis* passed through the Berkefeld filter in the first 200 c. c. of filtrate.

EXPERIMENT 2.

A flask of neutral beef broth was inoculated with culture Gp 3998 S and incubated at 37° C. for twenty-four hours. The culture showed well-marked clouding and a microscopic examination showed the organisms to be actively motile. Filtration was made through a Berkefeld laboratory cylinder No. 7. The amount of culture filtered was 100 c. c. and the time occupied in filtration was one and one-half hours. The filtration was conducted under a vacuum of 22 inches. The filtrate was collected in one portion and was immediately placed in the incubator. After twenty-four hours in the incubator the filtrate showed distinct clouding and agar plates revealed a pure culture of *B. cholerae suis*. A subculture made from the agar plates was cultivated on the different laboratory media. This culture was found to correspond with the original culture in all of its cultural characteristics. In this experiment *B. cholerae suis* passed through the Berkefeld filter in the first 100 c. c. of filtrate.

EXPERIMENT 3.

This experiment was practically a repetition of experiment 2 and the results obtained were the same. The filter used was a Berkefeld laboratory cylinder No. 7. The amount of culture filtered was 100 c. c., which required one and one-half hours to pass through the filter. The vacuum employed was 23½ inches. The entire filtrate was collected in one portion, as in the two preceding experiments. After forty-eight hours in the incubator the filtrate showed heavy clouding and agar plates revealed a pure culture of *B. cholerae suis*. A subculture was made from the agar plates and grown on the different laboratory media. This culture was found to correspond in all cultural characteristics with the original culture.

EXPERIMENT 4.

In this experiment the fractional method of filtration was used, the filtrates being collected in large sterile test tubes. In carrying out the experiment a twenty-four hour culture of Gp 3998 S, grown on neutral beef broth, was used. Filtration was made through a Berkefeld laboratory cylinder No. 7. The amount of culture filtered was 120 c. c. and the actual time consumed in the filtration was one hour and eighteen minutes. The suction varied from 20½ to 21½ inches. The details of the experiment are shown in the following table:

Table showing details of experiment 4.

Portion.	Amount.	Time consumed in passing filter.	Suction.	Result of incubation.
	c. c.	Minutes.	Inches.	
I.....	20	4	20½	Remained clear.
II.....	20	7	20½	Do.
III.....	20	10	20½	Do.
IV.....	20	12	21	Do.
V.....	10	7	21	Do.
VI.....	10	10	21	Do.
VII.....	10	11	21½	Clouded.
VIII.....	10	17	21½	Do.

The test tubes containing the filtrates were placed in the incubator immediately after filtration. At the end of twenty-four hours portions VII and VIII showed very slight clouding; other tubes remained clear. At the end of forty-eight hours portions VII and VIII showed increased clouding, while the others remained clear. Agar plates were made from portions VII and VIII and revealed pure cultures of *B. cholerae suis*. Subcultures from the agar plates were grown on the various laboratory media and found to correspond with the original culture. Portions I to VI were kept in the incubator for two weeks and remained perfectly clear. Hanging-drop preparations and agar plates from these portions failed to reveal any organisms.

In this experiment the Berkefeld cylinder held back the organisms until 100 c. c. of the culture had been filtered, but the first portion of 10 c. c. collected after 100 c. c. of culture had passed the filter showed *B. cholerae suis*.

EXPERIMENT 5.

In this experiment a twenty-six hour bouillon culture (H 2450 S) was used. The reaction of the beef broth in this instance was 1 per cent acid. The culture showed well-marked, uniform clouding, and a microscopic examination previous to filtration revealed active motility. Filtration was made through a Berkefeld cylinder No. 5 arranged for fractional filtration. The vacuum employed varied from 20 to 23 inches. It was the intention to filter several hundred cubic centimeters of the culture and collect the filtrate in separate

fractions, as in the preceding experiment, but the filtration proceeded very slowly and was discontinued at the end of forty-five minutes after 50 c. c. of culture had passed the filter. The filtrate was drawn off in a small sterile Erlenmeyer flask and placed in the incubator. At the end of eighteen hours the filtrate showed a distinct, uniform clouding and agar plates revealed a pure culture of *B. cholerae suis*.

In this experiment *B. cholerae suis* passed through a Berkefeld cylinder in the first 50 c. c. of filtrate.

SUMMARY OF EXPERIMENTS WITH BERKEFELD FILTERS.

The results of the experiments with Berkefeld filters are summarized in the following table:

Summary of Berkefeld filtrations.

No. of experiment.	Date begun.	Grade of filter.	Culture used.			Amount filtered.	Time occupied in filtration.	Vacuum.	Result.
			No.	Age.	Medium.				
1.....	1904. Feb. 28	No. 5..	Gp 3998 S...	Hrs. 24	Neutral beef broth.	c. c. 200	h. m. 1 30	Inches. 20	<i>B. cholerae suis</i> in filtrate.
2.....	Mar. 7	No. 7..	do.....	24	do.....	100	1 30	22	Do.
3.....	Mar. 9	No. 7..	do.....	24	do.....	100	1 30	23½	Do.
4.....	Mar. 15	No. 7..	do.....	24	do.....	120	1 18	20½-21½	<i>B. cholerae suis</i> in filtrate after 100 c. c. had passed filter.
5.....	1908. June 12	No. 5..	H 2450 S.....	26	+ 1 beef broth..	50	45	20-23	<i>B. cholerae suis</i> in filtrate.

DISCUSSION OF BERKEFELD FILTRATIONS.

In the foregoing experiments five Berkefeld cylinders, all of them new, were tested with bouillon cultures of *B. cholerae suis*, and in each instance the organism passed through the walls of the filter. In the case of one cylinder (experiment 4) the organism did not appear in the filter until 100 c. c. of the culture had been filtered. In one instance (experiment 5) the organism appeared in the first 50 c. c. of filtrate. With two of the cylinders (experiments 2 and 3) the organism passed through the walls of the filter in the first 100 c. c. of filtrate, and in the case of the remaining filter (experiment 1) the organism appeared in the first 200 c. c. of filtrate.

These results would indicate that in the case of liquid cultures or relatively heavy suspensions of small micro-organisms like *B. cholerae suis* the small laboratory Berkefeld filter can not be relied upon to keep back the organisms when any considerable amount of the material is filtered.

That the results obtained in these experiments conform with those obtained by other investigators who have tested the Berkefeld filter is shown by the following brief review of the literature relating to Berkefeld filters.

In 1902 Wherry^a found that the bacillus of pneumonia in guinea pigs, an organism somewhat shorter than, but very nearly as thick as, *B. cholerae suis*, would pass through the pores of a Berkefeld cylinder No. 5. Bouillon cultures one and three days old were used in these experiments, and the organism appeared in the filtrate after from 75 c. c. to 80 c. c. of the culture had been filtered.

Pfuhl^b in 1903 made a series of tests with four small Berkefeld cylinders and found that of these only one could be depended on with safety to deliver the first 100 c. c. of filtrate germ free. One of the filters gave 50 c. c. of germ-free filtrate, but no more; the other two filters failed to give even 50 c. c. of germ-free filtrate. The cultures used in these tests were *B. coli communis* and a vibrio of the same size as the cholera spirillum.

In a parallel series of experiments with large Berkefeld cylinders, which have thicker walls, Pfuhl found that 50 per cent of these filters were unable to restrain organisms approximating the size of *B. typhosus* and *B. dysenteriae*.

Novy and MacNeal^c in 1904 found that *Trypanosoma lewisi* could be passed through a Berkefeld filter.

Novy and Knapp^d in 1906 showed that even so large an organism as *Spirochaeta obermeieri*, which is 7 to 19 microns or more in length, will pass through the small Berkefeld filters under a pressure of 50 pounds.

Bulloch, Craw, and Atkin^e in 1908 tested ten Berkefeld filters with tap water and found that only one gave a sterile filtrate on the first day under a maximum pressure of 32.5 pounds to the square inch. The remaining nine yielded contaminated filtrates within fifteen minutes, or practically as soon as the filters were started.

EXPERIMENTS WITH PASTEUR-CHAMBERLAND FILTERS.

EXPERIMENT 1.

A flask of neutral beef broth was inoculated with culture Gp 3998 S and incubated for twenty-four hours. At the end of this time the culture showed well-marked clouding and a microscopic

^a Wherry, William B. Experiments on the permeability of the Berkefeld filter and the Pasteur-Chamberland bougie to bacteria of small size. *Journal of Medical Research*, vol. 8 (n. s., vol. 3), No. 2, pp. 322-328. Boston, Nov., 1902.

^b Pfuhl, E. Ergebnisse einer erneuten prüfung einiger kieseligur- und porzellanfilter auf keimdichtigkeit. *Festschrift zum 60th Geburtstag von Robert Koch*, pp. 75-86. Jena, 1903.

^c Novy, F. G., and MacNeal, Ward J. On the filtration of trypanosomes. *Michigan Academy of Science, Sixth Report*, p. 180. Lansing, 1904.

^d Novy, F. G., and Knapp, R. E. Studies on *Spirillum obermeieri* and related organisms. *Journal of Infectious Diseases*, vol. 3, No. 3, pp. 291-393. Chicago, May 18, 1906.

^e Bulloch, William, Craw, J. Anderson, and Atkin, E. E. On the relative efficacy of the Doulton, Berkefeld, and Brownlow filters. *Journal of Hygiene*, vol. 8, No. 1, pp. 63-69. Cambridge, Jan., 1908.

examination revealed active motility. The culture was then passed through an F bougie arranged to deliver into an ordinary side-arm suction flask. The amount of culture filtered was 200 c. c., the vacuum employed was 23 inches, and the time consumed in passing through the filter was thirty-five minutes. The entire filtrate was collected in one portion. When the filtration was complete the side-arm flask containing the filtrate was disconnected from the filter, plugged with a sterile cotton plug, and immediately placed in the incubator. At the end of eight days the filtrate was found to be perfectly clear, and a careful microscopic examination at this time failed to reveal any micro-organisms. A small portion of the filtrate was removed at this time by means of a sterile pipette and the remainder replaced in the incubator. A guinea pig was injected subcutaneously with 0.5 c. c. of the portion removed and showed no ill effects from the inoculation. The filtrate was kept in the incubator for six weeks and remained perfectly clear. The filtrate was again subjected to a careful microscopic examination before being discarded, and was apparently perfectly sterile.

EXPERIMENT 2.

A flask containing 400 c. c. of neutral beef broth was inoculated with culture Gp 3998 S and placed in the incubator for twenty-four hours. One-half of the culture (200 c. c.) was then passed through an F bougie and the other half through a B bougie, the two bougies being fitted to ordinary side-arm suction flasks. A vacuum of 21 to 22 inches was used, and the time required for the two filtrations, which were conducted simultaneously, was approximately forty-five minutes. The two side-arm flasks containing the filtrates were immediately placed in the incubator, where they remained for six weeks without showing any signs of bacterial growth. Before being finally discarded both filtrates were subjected to a careful microscopic examination, but no micro-organisms could be demonstrated in either hanging-drop or stained preparations.

EXPERIMENT 3.

A seven-day bouillon culture (Gp 4692 S) was used in this experiment. Filtration was made through a Pasteur-Chamberland B bougie. The filtration was discontinued after 100 c. c. of filtrate had passed the filter. The vacuum gauge recorded 20 inches during the filtration, which occupied thirty-seven minutes. The filtrate was collected in one portion and immediately placed in the incubator. After two weeks in the incubator the filtrate showed a very slight opalescence, but a careful microscopic examination failed to reveal any micro-organisms and the opalescence was attributed to a slight precipitate from the beef broth. In order to test further the ster-

ility of the filtrate, rabbits were injected as follows: Rabbit 1571 received 1 c. c. of filtrate intravenously; rabbit 1332, 3 c. c. intravenously; rabbit 1570, 5 c. c. intravenously; rabbit 1574, 5 c. c. intraperitoneally. These animals were kept under observation for several months and showed no ill effects whatever from the injections of filtrate.

The possibility now suggested itself that *Bacillus cholerae suis* might pass through the walls of the Pasteur-Chamberland filter in some form which would not develop *in vitro*, but which might develop within the animal body. In order to test this point collodion sacs were prepared according to the methods of Grubbs and Francis,^a of the United States Public Health and Marine-Hospital Service, and McCrae,^b of the Johns Hopkins Hospital. Sacs prepared according to each of these methods were filled with the filtrate and inserted into the abdominal cavities of rabbits. After an interval of several weeks the animals were killed and the sacs removed. The filtrate within the sacs showed no apparent clouding, and a microscopic examination of the contents of the sacs and cultures from the same failed to reveal any micro-organisms.

EXPERIMENT 4.

A forty-eight-hour bouillon culture (H 2199 S) was used in this experiment. Filtration was made through a Pasteur-Chamberland F bougie. In this experiment and in those which follow the fractional method of filtration was used, the filtrates being collected in separate portions in small sterile Erlenmeyer flasks, which were placed in the incubator as soon as the filtrations were completed.

Table showing details of experiment 4.

Portion	Amount.	Time occupied in passing filter.	Vacuum.	Result of incubation.
	c. c.		Inches.	
I.....	75	5 minutes.....	21	Clouding at 48 hours.
II.....	75	8 minutes.....	21	Do.
III.....	75	10 minutes.....	21	Clouding at 7 days.
IV.....	75	35 minutes.....	21	Clouding at 48 hours.
V.....	35	3 hours.....	21	Remained clear.
VI.....	10	Over night.....	No suction.	Do.

During the filtration of portions I, II, and III the bougie was entirely covered; during the filtration of portions IV, V, and VI it was only partly covered. Only about one-third of the filter was

^a Grubbs, S. B., and Francis, Edward. Laboratory technique. Collodion sacs. U. S. Treasury Department, Public Health and Marine-Hospital Service, Hygienic Laboratory, Bulletin 7. Washington, 1902.

^b McCrae, John. Notes upon the agglutinations obtained by intraperitoneal insertion of celloidin capsules containing bacilli and upon a mode of preparing such capsules. Journal of Experimental Medicine, vol. 5, No. 6, pp. 635-642. New York, Oct. 1, 1901.

covered with culture during the filtration of portion V, and this, together with the fact that the pores of the filter had become clogged, explains the length of time required for this portion to pass the filter.

After drawing off portion V the suction was disconnected and the apparatus left in place overnight. On the following morning it was found that approximately 10 c. c. of the culture had passed the filter, and this portion was drawn off and incubated along with the other fractions.

Portions I, II, and IV showed clouding at forty-eight hours. Microscopic examinations and agar plates showed in each case pure cultures of a micrococcus corresponding in morphology to *Staphylococcus pyogenes albus*, and probably due to outside contamination at the time these portions were drawn off. Guinea pigs weighing approximately 350 grams each were injected with 2 c. c. each of these portions and exhibited no ill effects from the injections.

Portion III became cloudy after one week in the incubator. Microscopic examination and agar plates revealed a pure culture of a micrococcus similar to that found in portions I, II, and IV. The injection of 2 c. c. of this portion into a guinea pig was without effect.

Portion V was kept in the incubator for ten days and remained perfectly clear. A portion of this filtrate was removed at the end of ten days by means of a sterile pipette, and a rabbit weighing 2,380 grams was given a subcutaneous injection of 10 c. c. without any subsequent ill effects. After drawing off the portion for the injection of the rabbit the remainder of the filtrate was inoculated with culture H 2199 S in order to see whether the filtrate would still furnish a suitable medium for the growth of *B. cholerae suis*. The inoculated portion was replaced in the incubator and showed well-marked clouding at twenty-four hours.

Portion VI was kept in the incubator for ten days and remained perfectly clear. It was then inoculated with culture H 2199 S, and showed well-marked clouding at twenty-four hours, thus demonstrating that *B. cholerae suis* would have grown in this filtrate had it passed through the pores of the filter.

The clouding of portions I, II, III, and IV in this experiment was due either to imperfect sterilization of the apparatus or to outside contamination at the time these portions were drawn off. That the clouding of these portions was not due to the passage of *B. cholerae suis* through the filter is shown by the fact that guinea pigs inoculated with these portions showed no ill effects from the inoculations.

It should be stated in this connection that the experiments described in this paper were conducted in a large general bacteriological work-room, where the conditions were more or less favorable for the outside contamination of the filtrates, owing to the constant passing of other workers and the impossibility of avoiding drafts and air currents.

EXPERIMENT 5.

In this experiment an eighteen-hour bouillon culture (H 2199 S) was used. The reaction of the bouillon in this experiment and those which follow was 1 per cent acid. Filtration was made through a Pasteur-Chamberland F bougie. The filtrate was collected in separate portions in small sterile Erlenmeyer flasks, which were placed in the incubator as soon as the filtration was completed. The bougie was kept entirely covered with culture during the filtration. The amount of culture filtered was 300 c. c.

Table showing details of experiment 5.

Portion	Amount.	Time occupied in filtration.	Vacuum.	Result of incubation.
	c. c.	Minutes.	Inches.	
I.....	75	2	20½	Clouded.
II.....	75	2	20½	Remained clear.
III.....	75	3	20½	Do.
IV.....	75	4	20½	Do.

Portion I showed clouding after forty-eight hours in the incubator. Microscopic examination and agar plates revealed a pure culture of a large spore-bearing organism resembling *Bacillus subtilis*. A guinea pig weighing 350 grams was injected with 2 c. c. of this portion but showed no ill effects from the injection. The organism noted in this flask was evidently due to outside contamination at the time the portion was drawn off.

Portions II, III, and IV were kept in the incubator for two weeks and remained perfectly clear. Two portions of 10 c. c. each were then removed from III and IV by means of sterile pipettes and injected into rabbits, as follows: Rabbit 2224 (weight 2,620 grams) received 10 c. c. of portion III; rabbit 2223 (weight 1,801 grams) received 10 c. c. of portion IV. The animals were kept under observation for several months, and showed no ill effects whatever from the injections.

After withdrawing portions from III and IV for the injection of rabbits, the remaining portions of these filtrates were inoculated with *B. cholerae suis* (culture H 2199 S) and returned to the incubator. After twenty-four hours both flasks showed well-marked clouding, and hanging-drop preparations and agar plates showed pure cultures of *B. cholerae suis*, proving that the absence of growth in these portions after filtration was not due to exhaustion of the bouillon.

EXPERIMENT 6.

Two flasks, each containing 400 c. c. of beef broth, were inoculated with *B. cholerae suis* (H 2199 S) and incubated for twenty-four hours. The contents of the two flasks were then poured together into a

large balloon flask and well mixed. The object of this was to give a sufficient quantity of culture to keep the bougie covered during the entire filtration. Filtration was made through a Pasteur-Chamberland F bougie, which was kept entirely covered by the culture throughout the filtration. The filtrate was collected in separate portions of 75 c. c. each in seven flasks, the total amount of culture filtered being 525 c. c.

Table showing details of experiment 6.

Portion.	Amount.	Time of filtration.	Vacuum.	Result of incubation.
	c. c.	Minutes.	Inches.	
I.....	75	2	20	Clouded.
II.....	75	3	20	Remained clear.
III.....	75	7	23-24	Do.
IV.....	75	9	23½-24	Do.
V.....	75	16	24-25	Do.
VI.....	75	19	25	Do.
VII.....	75	27	25	Do.

Portion I showed slight clouding at forty-eight hours, and a microscopic examination revealed a rather long, slender, nonmotile bacillus, apparently in pure culture. The flask was returned to the incubator and allowed to remain for six days, at the end of which time it showed well-marked, uniform clouding. Hanging-drop preparations and agar plates revealed the same bacillus that had been noted at forty-eight hours. A guinea pig, weighing approximately 350 grams, was injected with 2 c. c. of this portion after incubation for six days, but showed no ill effects as a result of the injection. The clouding of this portion was thus proven to be due to a contaminating organism and not to *B. cholerae suis*.

Portions II, III, IV, V, VI, and VII were incubated for twelve days, and remained perfectly clear. Rabbits were injected with portions VI and VII as follows: Rabbit 2222 (weight 2,500 grams) received 10 c. c. of portion VI; rabbit 2227 (weight 2,088 grams) received 10 c. c. of portion VII. These animals were kept under observation for several months, and remained perfectly well.

After removing portions for the injection of rabbits, the remaining portions of VI and VII were inoculated with *B. cholerae suis* (H 2199 S) and replaced in the incubator. Both flasks showed well-marked clouding at twenty-four hours, and microscopic preparations and agar plates revealed pure cultures of *B. cholerae suis*. This was done in order to prove that the bouillon had not been exhausted, but was still a suitable medium for the growth of *B. cholerae suis*.

EXPERIMENT 7.

In this experiment and in the one which follows Pasteur-Chamberland bougies were employed which had already been used in the preceding experiments. The bougies were cleansed according to

methods recommended by Lourens before being used the second time. Lourens, it should be stated, did not use new filters in all of his experiments, but adopted certain methods for cleansing his filters, and the two experiments which follow were designed with the view to determining whether the methods which he adopted for cleansing his filters could have had any effect on the permeability of the filters and so have influenced his results.

In carrying out experiment 7 an eighteen-hour bouillon culture of *B. cholerae suis* (H 2199 S) was used. The bougie used was a Pasteur-Chamberland F which had been used once before in experiment 2. Before being used the second time it was treated by the method which Lourens first used for cleansing his filters, as follows: The filter was first washed with cold tap water, boiled for ten minutes in 1 per cent hydrochloric acid, then boiled for ten minutes in a 3 per cent sodium carbonate solution, after which a liter or more of cold distilled water was passed through the filter until the filtrate showed only a faint alkaline reaction. The filter was sterilized by boiling for one-half hour in distilled water. The filtrate was collected in separate portions. The amount of culture filtered was 750 c. c.

Table showing details of experiment 7.

Portion.	Amount.	Time of filtration.	Vacuum.	Result of incubation.
	c. c.		Inches.	
I.....	75	1½ minutes.	20½	Remained clear.
II.....	75	2 minutes.	20 -18	Do.
III.....	75	3 minutes.	18 -21	Do.
IV.....	75	6 minutes.	21 -22½	Clouded.
V.....	75	9 minutes.	22½	Remained clear.
VI.....	75	12 minutes.	22½-21½	Do.
VII.....	75	16 minutes.	21½-22	Do.
VIII.....	75	1½ hours....	22	Do.
IX.....	150	Overnight....	No suction.	Do.

During the filtration of the first seven portions the bougie was entirely covered with culture; during the filtration of portion VIII the bougie was partly exposed. After portion VIII was drawn off the vacuum tubing was disconnected from the side-arm flask and the apparatus left in place overnight. On the following morning, after standing for eighteen hours, 150 c. c. of filtrate had collected in the side-arm flask. This portion, which passed through the filter during the night, was then drawn off and placed in the incubator, together with the other portions.

Portions I, II, III, V, VI, VII, VIII, and IX remained perfectly clear after eleven days in the incubator. Rabbits were then injected subcutaneously with portions VI and VII, as follows: Rabbit 2225 (weight 2,288 grams) received 10 c. c. of portion VI; rabbit 2226 (weight 2,185 grams) received 10 c. c. of portion VII. These animals were kept under observation for several months, and showed no ill effects from the inoculations.

Careful microscopic examinations were made of several of these filtrates, both in hanging-drop and in smear preparations stained with dilute carbol-fuchsin, and small, coccus-like bodies were noted, but cultures from the same portions gave negative results.

Portion IV showed clouding after four days, and agar plates revealed a pure culture of a medium-sized micrococcus corresponding in morphology with *S. pyogenes albus*. A guinea pig was inoculated with 2 c. c. of this portion after clouding had developed, and showed no ill effects from the inoculation. The growth in this flask was evidently due to outside contamination when the portion was drawn off. This is shown by the fact that all of the portions drawn off before this one, as well as all of those drawn off after it, were sterile.

Several of the filtrates after being incubated for eleven days with negative results were inoculated with *B. cholerae suis* and gave well-marked clouding at twenty-four hours, showing that the culture medium was still suitable for the growth of *B. cholerae suis*.

EXPERIMENT 8.

The culture used in this experiment was a twenty-four-hour bouillon of *B. cholerae suis* (H 2199 S). Filtration was made through a Pasteur-Chamberland F bougie which had been used once in experiment 6. The filter was cleansed according to the method finally adopted by Lourens as yielding the most satisfactory results. Following this method, the outside of the filter was first washed with cold tap water; a liter of cold distilled water was then passed through the filter; next a liter of potassium permanganate solution (1 gm. KMnO_4 , 6.5 gms. HCl , 1,000 c. c. water) was drawn through the filter; following the permanganate, a like amount of oxalic-acid solution (10 gms. oxalic acid to 1,000 c. c. water) was passed through the filter; hot water was next drawn through the filter until the filtrate was acid-free, and finally a liter of cold distilled water was passed through. Before being used the filter was sterilized in the usual manner by boiling for one-half hour in distilled water. The filtrate was collected in separate portions, the total amount of culture filtered being 525 c. c. During the filtration the entire filter was covered with the culture.

Table showing details of experiment 8.

Portion.	Amount.	Time of filtration.	Vacuum.	Result of incubation.
	c. c.	Minutes.	Inches.	
I.....	75	2½	23½	Remained clear.
II.....	75	6	23½	Do.
III.....	75	9	23½	Do.
IV.....	75	13	23½	Do.
V.....	75	22	23½ 17	Do.
VI.....	75	30	8	Do.
VII.....	75	40	8 10	Do.

Portions I to VII, inclusive, remained perfectly clear after ten days in the incubator, at the end of which time two rabbits were injected subcutaneously with portions VI and VII, as follows: Rabbit 2118 (weight 1,875 grams) received 10 c. c. of portion VI; rabbit 2119 (weight 1,892 grams) received 10 c. c. of portion VII. The animals were kept under observation for several months, but showed no ill effects from the injections. After withdrawing sufficient quantities of portions VI and VII for the injection of the rabbits, the remainders of these portions were inoculated with *B. cholerae suis* and showed well-marked clouding after twenty-four hours.

Portions I, II, III, IV, and V were left in the incubator for six weeks and remained perfectly clear. A microscopic examination of several of these portions revealed small bodies like those noted in experiment 7, but cultures from these portions yielded negative results.

EXPERIMENT 9.

A twenty-six hour bouillon culture of *B. cholerae suis* (H 2450 S) was used in this experiment. The filter used was a new Pasteur-Chamberland F bougie. The filtrate was collected in seven portions of 50 c. c. each, the total amount of culture filtered being 350 c. c. The bougie was kept covered with culture during the entire filtration.

Table showing details of experiment 9.

Portion.	Amount.	Time of filtration.	Vacuum.	Result of incubation.
	c. c.		Inches.	
I.....	50	40 seconds.....	23	Remained clear.
II.....	50	1½ minutes.....	23	Do.
III.....	50	2 minutes.....	23	Do.
IV.....	50	4½ minutes.....	23	Do.
V.....	50	6 minutes.....	20	Do.
VI.....	50	7½ minutes.....	20	Do.
VII.....	50	17 minutes.....	20	Do.

The filtrates were placed in the incubator immediately after filtration and showed no perceptible clouding after prolonged incubation.

Portions I, II, VI, and VII were left in the incubator for six weeks and remained perfectly clear. Cultures were made from these portions on various laboratory media, including Martin's serum-broth, with negative results.

Portion III was removed from the incubator at the end of two weeks and was found to be perfectly clear. This portion was subjected to a careful microscopic examination. In hanging-drop preparations small bodies having somewhat the appearance of micrococci were noticed. In preparations stained with dilute carbol-fuchsin which had been carefully filtered through double filters to remove all dirt and granules, small bodies were also noted; these bodies varied in size from minute points to bodies 0.6μ in diameter. No bacilli were noted in any of the preparations.

Cultures were made from this portion as follows: Two tubes of neutral agar, two tubes of neutral beef broth, and two tubes of Martin's bouillon with normal hog serum added were inoculated with 1 c. c. each, but none of these cultures showed any growth after two weeks' incubation. Martin's bouillon, with the addition of serum, was the medium upon which Nocard and Roux^a in 1898 first succeeded in cultivating outside of the animal body the organism of bovine pleuro-pneumonia, an organism which up to that time had resisted all attempts at cultivation and had been classed among the ultravisible viruses. Nocard and Roux employed Martin's bouillon with the addition of beef or rabbit serum, whereas in the present experiments hog serum was used as affording a more suitable medium for the possible growth of any living particles or bodies which might have passed through the pores of the filters.

Two hogs weighing from 30 to 40 pounds each were injected with portion III as follows: Hog 2351 received an intravenous injection in ear vein of 10 c. c. of portion III; hog 2352 received a subcutaneous injection in groin of 20 c. c. of portion III. These animals were kept under observation for several months and remained perfectly well.

In explaining his failure to produce hog cholera by the subcutaneous injection of cultures of *B. cholerae suis* and the high degree of virulence which the blood of animals sick of hog cholera exhibits when injected subcutaneously, Theobald Smith^b advanced the theory that the blood coagulates in the connective tissues and serves as a food for the bacilli which it incloses and at the same time protects them against the action of the leucocytes. With this theory in mind, and in order to determine whether the coccus-like bodies noted in this filtrate possessed any significance—that is, whether they were capable of further development in the animal body—the following experiment was carried out: Ten cubic centimeters of blood was drawn from the tail of a healthy hog under aseptic conditions and collected in a large sterile test tube, the interior of which had received a coating of sterile olive oil to prevent coagulation. To this blood was immediately added 10 c. c. of portion III. The mixture of blood and filtrate was then drawn up into a sterile syringe and at once injected into the groin of a healthy hog (No. 2354) before coagulation had time to take place. Now, if the coccus-like bodies noted in this filtrate had been capable of developing into bacillary forms, as Lourens claimed for those which he observed, they were certainly afforded in this experiment, according to Smith's theory, a good opportunity to develop within the animal body. This hog, however, was kept under observation for several months and remained perfectly well.

^aNocard, E. I. E., and Roux. Le microbe de la péripneumonie. Annales de l'Institut Pasteur, tome 12, No. 4, pp. 240-262, Paris, Apr. 25, 1908.

^bHog Cholera: Its History, Nature, and Treatment. United States Department of Agriculture, 1889.

Portion IV was removed from the incubator at the end of three weeks. Hanging-drop and smear preparations stained with dilute carbol-fuchsin showed bodies similar to those noted in portion III, but no bacilli. Two rabbits, weighing approximately 2,000 grams each, were injected as follows: Rabbit 2306 received an intravenous injection of 3 c. c. of portion IV; rabbit 2307 received a subcutaneous injection of 10 c. c. of portion IV. Both animals remained well and showed no ill effects as a result of the injections.

Portion V was removed from the incubator at the end of four weeks. Microscopic examination revealed the same bodies noted in portions III and IV, but there had been no apparent multiplication of these bodies and no bacilli were observed. Two rabbits, weighing approximately 2,300 grams each, were injected as follows: Rabbit 2301 received an intravenous injection of 3 c. c. of portion V; rabbit 2308 received a subcutaneous injection of 10 c. c. of portion V. Both animals remained well, showing no ill effects whatever from the injections.

EXPERIMENT 10.

In connection with his culture experiments Lourens states that the addition of an equal quantity of serum to bouillon furnishes a fluid which considerably increases the permeability of filters of the Berkefeld and Pasteur-Chamberland types. In order to test the correctness of this statement with regard to filters of the Pasteur-Chamberland type the following experiment was carried out:

A flask containing 250 c. c. of bouillon was inoculated with *B. cholerae suis* (H 2228 S) and incubated for forty-four hours. An equal amount (250 c. c.) of horse serum was then added to the bouillon culture and the mixture thoroughly shaken. Filtration was made through a new Pasteur-Chamberland F bougie. The filtrate was collected in seven portions of 70 c. c. each, the total amount of culture filtered being 490 c. c.

Table showing details of experiment 10.

Portion.	Amount.	Time of filtration.	Vacuum.	Result of incubation.
	c. c.	Minutes.	Inches.	
I.....	70	1	28	Filtrate free from <i>B. cholerae suis</i> .
II.....	70	1	28	Do.
III.....	70	1½	28	Do.
IV.....	70	3	28	Do.
V.....	70	7	28	Do.
VI.....	70	27	28	Do.
VII.....	70	60	28	Do.

After twenty-four hours in the incubator the filtrates all showed a slight opalescence, but there was no apparent clouding of the medium. After one week in the incubator the opalescence noted at the end of twenty-four hours had not increased and there was still no clouding of the filtrates.

Cultures were made from portions I, II, V, VI, and VII on various laboratory media, including Martin's serum-broth, with negative results.

Portions III and IV were removed from the incubator at the end of one week and subjected to a careful microscopic examination. Smear preparations stained with dilute carbol-fuchsin showed a finely granular material and occasional small coccus-like bodies like those noted in experiment 9. Cultures were made from each of these portions by adding 10 c. c. to flasks containing 400 c. c. each of beef broth. Rabbits were then given subcutaneous injections as follows: Rabbit 2428 (weight 2,160 grams) received 10 c. c. of portion III; rabbit 2429 (weight 2,935 grams) received 10 c. c. of portion IV.

After the removal by means of sterile pipettes of the portions used for the inoculation of cultures and rabbits, portions III and IV were then inoculated with *B. cholerae suis* (H 2228 S) and replaced in the incubator. Both portions showed well-marked clouding at the end of twenty-four hours, and microscopic examination revealed actively motile forms of *B. cholerae suis*, thus proving that the medium was not unfavorable to the growth of the bacillus.

The two flasks of bouillon which were inoculated with 10 c. c. each of portions III and IV failed to show any growth upon prolonged incubation, and the two rabbits inoculated with like amounts of the same portions showed no ill effects as a result of the inoculations.

The opalescence noted in the filtrates was evidently due to a slight precipitation of albuminous material from the blood serum.

This experiment would show that in the case of the Pasteur-Chamberland filter the addition of serum to the material filtered does not affect the permeability of the filter.

SUMMARY OF EXPERIMENTS WITH PASTEUR-CHAMBERLAND FILTERS.

The results of the experiments with Pasteur-Chamberland filters are summarized in the following table:

Summary of Pasteur-Chamberland filtrations.

No. of experiment.	Date begun.	Culture.		Grade of filter.	Amount of culture filtered.	Time occupied in filtration.	Vacuum.	Result of incubation.
		Number.	Age.					
1.....	Feb. 24, 1904	Gp 3998 S.	24 hours ..	F	c. c. 200	h. m. 35	Inches. 23	<i>B. cholerae suis</i> absent.
2.....	Mar. 2, 1904	do.....	do.....	F and B	200	45	21-22	Do.
3.....	June 6, 1905	Gp 4032 S.	7 days	B	100	37	20	Do.
4.....	Nov. 29, 1907	II 2199 S.	2 days	F	335	3 58	21	Do.
5.....	Nov. 30, 1907	do.....	18 hours	F	300	11	20½	Do.
6.....	Dec. 3, 1907	do.....	24 hours	F	525	1 23	20 25	Do.
7.....	Dec. 5, 1907	do.....	18 hours	F	600	2 44	18 22½	Do.
8.....	Dec. 7, 1907	do.....	24 hours	F	525	2 24	8-23½	Do.
9.....	June 12, 1908	II 2450 S.	26 hours	F	350	39	20-23	Do.
10.....	Aug. 24, 1908	II 2228 S.	44 hours	F	490	1 40	28	Do.

a The portions which filtered through overnight under atmospheric pressure are not included in this table.

DISCUSSION OF PASTEUR-CHAMBERLAND FILTRATIONS.

In the experiments just described, nine Pasteur-Chamberland filters were tested with bouillon cultures of *B. cholerae suis* under an average vacuum of 20 to 25 inches. Five different strains of *B. cholerae suis* were used, and the cultures filtered varied in age from eighteen hours to seven days. The amount of culture filtered varied from 100 to 750 c. c., and in not a single instance could *B. cholerae suis* be detected in the filtrates.

In testing the filtrates for *B. cholerae suis* one or more, sometimes all, of the following methods were used: (1) Incubation of the filtrate, either entire or in fractions, for a considerable period of time; (2) the injection of large amounts of the filtrate into susceptible animals; (3) the introduction of collodion sacs containing filtrate into the abdominal cavities of susceptible animals; (4) microscopic examination, in both hanging-drop and stained preparations; (5) subcultures on various media favorable to the growth of *B. cholerae suis*. By none of these methods, however, could *B. cholerae suis* be detected in any of the filtrates, and the conclusion therefore seems justified that the organisms were completely restrained by the filters.

In view of the fact that all of the nine filters tested proved equally efficient in preventing the passage of *B. cholerae suis*, we may further conclude that the Pasteur-Chamberland filter may be depended on with safety to furnish as much as 500 to 600 c. c. of bacteria-free filtrates from bouillon cultures or correspondingly heavy suspensions of organisms approximating in size to *B. cholerae suis*. As it has been shown that bacteria are capable of growing through the walls of bacterial filters, it is best to limit the duration of the filtration to two or three hours. In two of the experiments recorded in this paper (Chamberland experiments 4 and 7) the apparatus was left in place and the portions which passed through the filters overnight were collected and incubated. These portions remained sterile, showing that in two instances at least *B. cholerae suis* did not grow through the walls of the Chamberland F filter in sixteen hours. Just how long it will require for different bacteria to grow through the Chamberland filter is a point which has not been fully investigated.

Although it does not seem probable in view of the perfect efficiency exhibited by the Pasteur-Chamberland filters in the experiments just described, it is nevertheless possible that an imperfect bougie may occasionally go out on the market, and for this reason it is important in all filtration experiments where bacteria-free filtrates are desired, even where the Pasteur-Chamberland filter is used, that all filtrates as well as filters be carefully tested.

Even if the experiments described in Bulletin 72 of this Bureau, previously mentioned, to which Lourens takes exception, had not

been sufficiently and carefully checked at the time they were made, the experiments with Pasteur-Chamberland filters described in the present paper would go far to prove that the filtrates employed in Bulletin 72 were absolutely free from *B. cholerae suis*, for in nearly all of the filtration experiments described in that bulletin the Pasteur-Chamberland type of filter was used almost exclusively, a new filter being used for each experiment, and the amount of filtrate collected never exceeded 500 cubic centimeters.

Lourens, however, criticises the filtration experiments described in Bulletin 72 on the ground that in testing the sterility of the filtrates cultures were made from the filtrates immediately after filtration. He claims that his experiments show that cultures made from filtrates immediately after filtration will almost always remain sterile, even though they be kept at incubator temperature for several weeks. This he explains on the ground that there may be so few organisms in the filtrate that they are missed in taking out portions for cultures. In making this criticism he apparently leaves out of consideration a point upon which he lays great emphasis in his conclusions, namely, the formation of granules sufficiently small to pass through the walls of a porcelain filter and capable of developing in the filtrates into *B. cholerae suis*. Now, if these granules be sufficiently small to pass through the walls of a porcelain filter, they should be present in the filtrates in considerable numbers and could hardly be missed in taking out portions for cultures. But aside from this apparent contradiction, we do not consider Lourens's criticism well founded, for as previously stated, repeated tests of the filtrates described in Bulletin 72 showed that cultures made with several times the amount of filtrate used for the inoculation of hogs did not show *B. cholerae suis* in a single instance, and guinea pigs and rabbits were unaffected by amounts sufficient to cause the death of hogs.

In the experiments described in the present paper the filtrates were held at incubator temperature for from one to two weeks before they were tested on animals, and supposing that only a very few organisms had passed through the filters into the filtrates these would certainly have had ample time to develop before the filtrates were tested. Where the entire filtrate was held at incubator temperature for a considerable time, as just explained, it did not seem necessary to make cultures from the filtrates in every instance, as it was repeatedly proven by subsequent inoculation that the filtrates themselves afforded a suitable medium for the growth of *B. cholerae suis* had this organism succeeded in passing through the walls of the filter.

Pasteur-Chamberland filters, which were subjected to a single cleansing according to the methods recommended by Lourens, were apparently unimpaired in efficiency, but it does not seem unlikely that repeated cleansings by these methods might in time affect the

pores of the filter and render it more permeable. Indeed, it is hard to explain the results of Lourens's Pasteur-Chamberland filtrations on any other ground unless it be that he used excessively high pressures in carrying out his filtrations. Lourens describes six experiments with bouillon cultures of *B. cholerae suis* in which Pasteur-Chamberland filters were used, and in four out of the six experiments *B. cholerae suis* appeared in the filtrates. In none of these six experiments does he appear to have used new filters, nor does he state how often his filters had been previously used and cleansed, but presumably they had been used a number of times. Lourens also fails to describe the manner in which he arranged his filters, whether for air pressure or vacuum, and nowhere in the course of his article does he record the amount of pressure or vacuum employed. It would appear, however, from the brief paragraph which he devotes to the technique of his filtration experiments that he made use of pressure in carrying out his filtrations, his filters being so arranged that air pressure could be applied to the fluid surrounding the filter and the fluid thus forced through the walls of the filter instead of being drawn through by means of suction. That he employed considerable pressure in carrying out his experiments is indicated from his statement that he experienced no difficulty in passing 500 c. c. of undiluted blood serum through a Berkefeld filter (size of filter not stated) in fifteen minutes or less, and that it required but little longer to pass the same amount of serum through a Chamberland F filter.

As this last statement in regard to the filtration of blood serum is so at variance with our own experience and the experience of other investigators who have found that blood serum filters with considerable difficulty, we have tested a number of Berkefeld and Chamberland filters with distilled water and blood serum, in order to determine their approximate rates of filtration.

RATE OF FILTRATION OF BERKEFELD AND PASTEUR-CHAMBERLAND FILTERS.

Employing a vacuum of 29 inches, it required about fifteen minutes to pass 500 c. c. of distilled water through a No. 7 Berkefeld laboratory cylinder, about ten minutes to pass the same amount through a Chamberland F filter, and from fifteen to twenty minutes to pass a like amount through a Chamberland B filter. With clear, limpid hog serum, which was first passed through a large No. 2 Berkefeld cylinder, in order to free it from all red blood cells, it required over three hours to pass 500 c. c. through a Berkefeld laboratory cylinder No. 7 under a vacuum of 29 inches and over four hours to pass 300 c. c. through a Chamberland F filter under the same vacuum. In the case of both filters the filtration of the blood serum proceeded with progressive slowness, as was shown by collecting the filtrates in separate portions of 100 c. c. each. In the case of the Berkefeld filter

the first 100 c. c. of serum passed the filter in six minutes, the second in nine minutes, the third in twelve, and the fourth in thirty minutes, whereas the fifth portion of 100 c. c. required two and one-half hours to pass the filter, at the end of which time the filter had virtually ceased to act. In the case of the Chamberland filter the first 100 c. c. of serum passed the filter in fifteen minutes, the second in one hour, while the third portion of 100 c. c. required three hours to pass the filter, at the end of which time the filter had become completely clogged and filtration had practically ceased.

In view of these results, it is difficult to see how Lourens succeeded in passing 500 c. c. of blood serum through a Chamberland F filter in fifteen minutes, unless he used very high pressure, and if he used such pressure it is conceivable that in his filtration experiments the clouding of his filtrates from *B. cholerae suis* was due to the fact that he actually forced the organisms themselves through the walls of his filters.

GRANULE FORMATION IN CULTURES OF *B. CHOLERAË SUIS*.

With regard to the presence of granules in cultures of *B. cholerae suis*, Lourens states that only certain strains possess this property of breaking up into granules, but in those strains which are characterized by granule formation the peculiarity is a fixed one and persists after the organism has been cultivated on the different culture media and also after it has been passed through the animal body. He states that in his experiments only those cultures which were characterized by granule formation and polar staining passed through the filters, his theory being that the granules are sufficiently small to pass through the pores of the filters, and after passing through the filters these granules are capable of developing in the filtrates into the characteristic bacillary forms. He describes the granule formation as giving rise to coccus-like forms which may have the appearance of constricted bacilli or cocci lying in close juxtaposition, and now and then as three coccus-like bodies lying free or surrounded by a zone resembling a capsule.

Three of the five cultures used in the experiments described in the present paper were carefully examined for granules like those described by Lourens. The stock cultures of two of the strains experimented with were unfortunately lost in moving the laboratory and could not be examined for granules. In examining the cultures for granules, smear preparations were made from bouillon cultures and from the water of condensation of agar cultures. The films were carefully fixed by means of formalin and methyl alcohol without the aid of heat, so as to avoid any possible distortion from overheating, and were stained with dilute carbol-fuchsin, as recommended by Lourens. In the three cultures examined polar staining was observed

in all and granular forms were noted which corresponded in every particular with those described and pictured by Lourens. We must admit, therefore, the correctness of Lourens's observation as to the formation of granules in cultures of *B. cholerae suis*, and it is also possible that these granules may pass through the pores of an unglazed porcelain filter; but in view of the results of our Pasteur-Chamberland filtrations, where the filtrates were proven in every instance to be free from *B. cholerae suis*, although at least three of the cultures experimented with showed granule formation, we must conclude that the granules upon which Lourens lays so much stress are incapable of developing into the characteristic bacillary forms of *B. cholerae suis* and that they possess no significance in filtration experiments with this organism.

In our experiments with the Berkefeld filter, where *B. cholerae suis* developed in the filtrates, we believe that the organisms themselves passed through the walls of the filter.

SUMMARY OF RESULTS.

When bouillon cultures of *B. cholerae suis* were filtered through the smaller Berkefeld laboratory filters it was found that after a time—that is, after a certain amount of culture had been filtered—the organisms appeared in the filtrates. With a vacuum of 20 to 25 inches of mercury not more than 100 c. c. of bacteria-free filtrate could be obtained with these filters. When bouillon cultures of *B. cholerae suis* were filtered through Pasteur-Chamberland filters (F and B), the organisms did not appear in the filtrates in a single instance, although as much as 600 c. c. of culture was filtered in one instance. With a vacuum of 20 to 25 inches of mercury, the Pasteur-Chamberland filters (F and B) can be depended on to furnish from 500 to 600 c. c. of bacteria-free filtrate from bouillon cultures of *B. cholerae suis* when the time consumed in filtration does not occupy more than two hours.

Beef broth or bouillon is apparently unaltered by passage through a Berkefeld or Pasteur-Chamberland filter, and the absence of growth in filtrates from bouillon cultures of *B. cholerae suis* can not be explained on the supposition that filtration effects an alteration in the bouillon which renders it unfit for the growth of the organism. The addition of an equal volume of horse serum to a bouillon culture of *B. cholerae suis* did not facilitate the passage of the organisms through the Pasteur-Chamberland filter.

Rabbits were injected subcutaneously with 10 c. c. of filtered culture, and other rabbits were injected intravenously and intraperitoneally with 5 c. c. of filtered culture, but none of these animals showed any ill effects from the injections. Hogs weighing from 30 to 40 pounds were injected subcutaneously with 20 c. c. of filtered culture and intravenously with 10 c. c. of filtered culture, but were not

rendered sick thereby. Collodion sacs containing filtered culture were placed in the abdominal cavities of rabbits, but remained sterile.

Granules were noted in cultures of *B. cholerae suis* and in the filtrates from these cultures, but these granules did not develop in the filtrates nor in subcultures made from these filtrates. These granules were also shown to be incapable of development in the bodies of rabbits and hogs.

CONCLUSIONS.

In view of the results stated above, we must conclude—

1. That Pasteur-Chamberland filters F and B effectually prevent the passage of *B. cholerae suis*.

2. That the smaller Berkefeld laboratory cylinders vary in permeability.

3. That certain of the Berkefeld laboratory cylinders will prevent the passage of *B. cholerae suis* when a limited amount of material is filtered.

4. That the granules noted in cultures of *B. cholerae suis* have no significance in filtration experiments with this organism.

5. That in the filtration experiments described in Bulletin 72 the filtrates employed did not contain *B. cholerae suis*.

6. That hog cholera is due to an ultra-visible virus sufficiently small to pass through the pores of the Chamberland filter.



Issued June 10, 1909.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY.—BULLETIN 114.
A. D. MELVIN, CHIEF OF BUREAU.

THE INFLUENCE OF ACIDITY OF CREAM
ON THE FLAVOR OF BUTTER.

BY

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1909.

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LETTER OF TRANSMITTAL.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY,
Washington, D. C., March 29, 1909.

SIR: I have the honor to transmit herewith, with the recommendation that it be published as a bulletin of this Bureau, a paper entitled "The Influence of Acidity of Cream on the Flavor of Butter," by L. A. Rogers and C. E. Gray.

The deterioration in quality and the development of objectionable flavors in butter kept in cold storage cause considerable loss to the trade, and the causes and nature of these changes have not been understood. The Dairy Division of this Bureau has been making a study of these problems during the past three years, and the present paper reports investigations in which quantities of butter were made from cream of varying degrees of acidity and stored at different temperatures. It is believed that the results will be of practical value to the butter manufacturers of the country.

The authors wish to express their appreciation of the services of the various persons whose cooperation has made this work possible. They are especially indebted to the director and members of the staff of the University of Wisconsin Agricultural Experiment Station, who kindly allowed the use of their creamery and laboratories for part of the work. They are also under obligations to the manager of the creamery at Bloomer, Wis., and to the several persons who kindly scored the butter.

Respectfully,

A. D. MELVIN,
Chief of Bureau.

HON. JAMES WILSON,
Secretary of Agriculture.

CONTENTS.

	Page.
Introduction.....	7
Experiments with butter made from cream with varying acidity.....	8
Discussion of results	11
Determinations of bacteria in the butter.....	12
Possible action of enzymes in deteriorating high-acid butter.....	15
Influence of lactic acid on flavor of butter.....	17
Result of producing acidity with other acids than lactic acid.....	17
The manufacture of commercial butter from sweet cream	18
Conclusions.....	21

THE INFLUENCE OF ACIDITY OF CREAM ON THE FLAVOR OF BUTTER.

INTRODUCTION.

Although much has been written about butter, there is yet very little known about the changes taking place in its chemical composition, the relation of these changes to the changes in flavor, the causes which produce them, or the factors which control their progress. It becomes necessary, therefore, to work out one by one the various conditions which cause or control the changes in the composition and flavor of butter. The difficulty of controlling conditions exactly and at the same time making normal butter renders it hard to determine the part played by any one factor. However, it is only by limiting the variations so far as the circumstances will permit to one factor that any definite conclusions can be reached.

A factor in the manufacture of butter, the variations of which can be controlled, is the acidity developed in the cream. This factor has long been recognized as important in determining the flavor of butter and as having a decided influence on the constancy with which butter retains its desirable flavors in storage.

It is generally taught by instructors and writers on dairy subjects that to produce good butter it is necessary to develop a certain amount of acid in the cream. The reason for this is twofold—first, to develop a desirable flavor, and, second, to improve the keeping quality by suppressing the undesirable bacteria. McKay and Larsen^a state that in the ripening of cream the lactic-acid bacteria suppress other bacteria which, if carried into the butter, would produce undesirable changes. It is recognized, however, that if the fermentation is carried too far the keeping quality of the butter is injured. McKay and Larsen also state that in overripened cream undesirable bacteria may gain the ascendancy and cause deterioration of the butter.

Michels^b states:

It has been found that butter with the best keeping quality is obtained from well-ripened cream. It is true, however, that butter made from cream that has been ripened a little too far will possess very poor keeping quality. An acidity of 0.5 per cent should be placed as the limit when good keeping quality is desired.

^aGeorge L. McKay and C. Larsen. *Principles and Practice of Butter-making*. P. 194. 1906.

^bJohn Michels. *Creamery Butter Making*. P. 70. 1904.

These statements by recognized authorities may be taken as an expression of the opinion of instructors, investigators, and butter-makers in general. This opinion seems to be based, not on the results of actual experiment, but on experience in buttermaking. The little experimental evidence available on this question is conflicting and inconclusive. Patrick, Leighton, and Bisbee,^a and Patrick, Leighton, and Heileman^b concluded that butter made from sweet cream retained its flavor better than butter made from sour cream. The opposite conclusion was reached by Dean.^c Unpasteurized cream was evidently used in these experiments.

The Dairy Division of the Bureau of Animal Industry therefore planned to include in a general investigation of the changes in storage butter a scientific and carefully controlled study of the influence of the acidity of the cream on the keeping quality of the butter in order to determine, if possible, the proper conditions under which butter intended for storage should be made. Obviously, results obtained from pasteurized cream could not properly be applied to butter made from unpasteurized cream, and vice versa. Therefore the investigation was planned to include butter made from both pasteurized and unpasteurized cream with varying degrees of acidity.

EXPERIMENTS WITH BUTTER MADE FROM CREAM WITH VARYING ACIDITY.

The first lot of butter in this investigation was made in the creamery of the University of Wisconsin dairy school at Madison in the summer of 1906. Owing to the fact that it was necessary to obtain part of the cream from another creamery it was impossible to control conditions as exactly as was desired, since in some cases the ripening developed more than had been planned.

The entire lot of cream was mixed; one half the quantity was pasteurized in a Farrington pasteurizer at 180° F.; the other half was not pasteurized. After the pasteurization of the first half the two lots of cream were treated in the same way. One-fourth of each lot was cooled and then churned as soon as possible. To the remaining cream was added 14 per cent of the starter in use at the creamery. One-third of this lot was cooled and churned as soon as possible. The remaining cream was allowed to ripen to about 0.45 per cent,^d

^a G. E. Patrick, F. A. Leighton, and D. B. Bisbee. Sweet versus Sour Cream Butter. Iowa Agricultural Experiment Station, Bulletin 18, pp. 478-487. 1892.

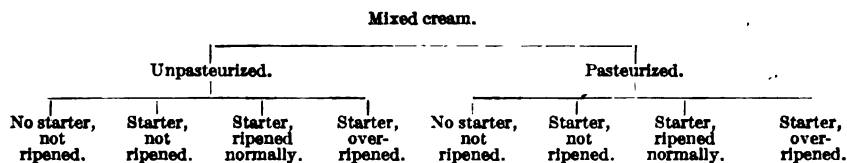
^b G. E. Patrick, F. A. Leighton, and W. H. Heileman. Sweet versus Ripened Cream Butter. Iowa Agricultural Experiment Station, Bulletin 21, pp. 788-791. 1893.

^c H. H. Dean. Experiments in Creaming and Butter Making. Ontario Agricultural College and Experimental Farms, Twenty-first Annual Report (for 1895), pp. 60-66. Toronto, 1896.

^d In this paper acidity is expressed as per cent of lactic acid.

when it was divided, one half cooled and churned, and the other half allowed to stand forty-eight hours after separation.

The mode of procedure may be illustrated by the following diagram:



The acidity of the overripened cream developed very little above that of the normally ripened cream. The acidity of the overripened pasteurized cream at time of churning was 0.52 per cent. The acidity of the corresponding unpasteurized cream was 0.47 per cent. The salt and moisture contents were controlled as closely as possible and showed no variations which could be expected to influence the results, except in the case of the unpasteurized cream churned without starter. In this case the salt was 1.81 per cent. The butter, which will be designated as Lot I, was packed in 20-pound tubs and held seven days in the creamery refrigerator, when it was scored by Mr. J. G. Moore and then shipped by refrigerator freight to the cold-storage rooms in Chicago. Two tubs of each lot were stored at temperatures of 32°, 10°, and -10° F. The storage rooms were held at these temperatures with very little variation.

In the following summer a quantity of butter (Lot II) was made at Albert Lea, Minn., by the same method. This butter was made from milk received in the creamery in one day. Half of this cream was pasteurized in a regenerative Jensen pasteurizer at 170° F. The various lots were churned at the following acidities:

Unpasteurized:	Per cent.
No starter.....	0.13
Starter added.....	.37
Ripened.....	.51
Overripened.....	.86
Pasteurized:	
No starter.....	.13
Starter added.....	.16
Normally ripened.....	.51
Overripened.....	.68

The starter used in this cream was not very active and the acidity developed slowly in the pasteurized cream; otherwise this butter could be taken as representative of butter made under normal commercial conditions. The butter was packed in sealed 10-pound tin cans and stored, as was the Madison butter, at 32°, 10°, and -10° F.

The butter of Lot I was scored after six months in storage by Messrs. Credicott, Smarzo, Kieffer, and White, and after nine months by the

three first named. Each man scored independently and without knowledge of the history of the butter. The average scores of the Madison butter were as follows:

TABLE 1.—Average scores of butter of Lot I.

Method.	Storage temperature.	Score when fresh.	Score after six months' storage.	Score after nine months' storage.
Unpasteurized:				
	° F.			
No starter, no ripening.....	-10	92	90.6	91.3
	10		91	91.6
	32		87	86.6
Starter, no ripening.....	-10	93.5	89.5	90
	10		90	92
	32		86.5	86
Starter, normal ripening.....	-10	96	91	91.3
	10		90	91
	32		86.5	85.3
Starter, overripening.....	-10	92	90.05	92.6
	10		90.2	No tub.
	32		87.2	87.5
Pasteurized:				
No starter, no ripening.....	-10	97	91.3	92.3
	10		91.3	92
	32		90.6	89.3
Starter, no ripening.....	-10	96.5	91.18	92
	10		91.37	92
	32		88.3	89
Starter, normal ripening.....	-10	95	89	91.8
	10		87.5	90.3
	32		85.6	84
Starter, overripening.....	-10	92.5	85.5	90.3
	10		85.1	84.3
	32		81.75	82

The butter of Lot II stored at 10° F. was scored after four months by Mr. Credicott, and all the butter was scored after eight months by Messrs. Kieffer and Smarzo. In the latter scoring a number of grades with a definite score were arranged and the packages grouped accordingly. In all cases a separate package was used for each scoring.

TABLE 2.—Average scores of butter of Lot II.

Method.	Storage temperature.	Score after four months.	Score after six months.
Unpasteurized:			
	° F.		
No starter, churned sweet.....	-10		90.
	10	90. Rancid.....	90.
	32		87. Cheesy.
Starter, not ripened.....	-10		90.
	10	90. Rancid.....	90.
	32		90.
Starter.....	-10		90.
	10	90. Cold storage.....	82. Fishy.
	32		82. Fishy.
Starter, overripened.....	-10		84. Curdy, cheesy.
	10	89. Bad.....	82. Curdy, very poor.
	32		82. Curdy, very poor.
Pasteurized:			
No starter, churned sweet.....	-10		92.
	10	92.5.....	90.
	32		91.
Starter, not ripened.....	-10		91.
	10	92.....	91.
	32		91.
Starter, normal ripening.....	-10		91.
	10	91. Cold storage.....	91.
	32		90.
Starter, overripened.....	-10		81. Curdy, cheesy.
	10	88. Bad.....	82. Curdy, very poor.
	32		82. Curdy, very poor.

A third lot of butter (Lot III) was made, as before, from one lot of cream, one-half of which was pasteurized at 180° F. After taking out one-fourth of each half to be churned sweet, 15 per cent of starter was added. The different portions of this lot of cream were churned at the following acidities:

Unpasteurized cream:	Per cent.
No starter.....	0.18
Starter added.....	.32
Ripened.....	.54
Overripened.....	.64
Pasteurized cream:	
No starter.....	.16
Starter added.....	.32
Ripened.....	.59
Overripened.....	.70

This butter was packed in sealed tin cans. One can from each lot was sent to New York, where it was scored when twenty days old by Mr. Kieffer. The remainder was stored at 10° F. and was again scored by Mr. Kieffer at the end of five months. The scores are shown in the following table:

TABLE 3.—*Scores of butter in Lot III.*

Method.	Twenty days old.	After five months' storage at 10° F.
Unpasteurized:		
No starter.....	85. Strong.....	90.
Starter added.....	90.	90.
Ripened.....	88. Oily.....	80. Very fishy.
Overripened.....	85.	88.
Pasteurized:		
No starter.....	93.	91.
Starter added.....	95.	90.
Ripened.....	90. Slight metallic.....	87.
Overripened.....	87. Metallic.....	87.

DISCUSSION OF RESULTS.

The score of the fresh butter of Lot I immediately before it went into storage indicates that while there were some differences all of the butter was at least fairly good, while the average of the scores made after the butter had been in storage shows marked differences in a few cases. The comments of the scorers show that the butter made from unripened unpasteurized cream always developed a cheesy or rancid flavor. The butter made from ripened cream, both pasteurized and unpasteurized, developed cold-storage, fishy, and other flavors typical of storage butter. In all cases the overripe butter showed marked deterioration. The butter made from pasteurized cream without starter usually retained its flavor with little or no change. Even at 32° F., where all the ripened butter showed decided changes, the sweet-cream butter deteriorated very little. The score of the sweet-cream butter in the Madison lot was decreased by a "woody"

or "fruity" flavor which was noticeable at the top and sides of the package. In the opinion of the judges, this flavor was due to some extraneous cause.

The difference between butter made from pasteurized sweet cream and that from ripened cream, both pasteurized and unpasteurized, became very marked after holding in a warm room for a short time. Butter made from pasteurized cream with starter added, after the so-called Le Clair or Credicott method, retained its fresh flavor better than the ripened-cream butter, but was not quite equal in keeping quality to that made from sweet pasteurized cream.

In making deductions from results obtained by the usual method of scoring butter, allowance must be made for certain variations due to the sense of taste, which is not adapted to expression in mathematical terms. However, after making allowance for the personal tastes and variations that can not be avoided in butter scoring, it is evident from the results shown in the tables that some factor having a deleterious influence on the butter was developed with the ripening of the cream. The action of this factor is especially evident in the cream allowed to stand beyond the usual ripening period, although it is not necessarily accompanied by a marked increase in acidity, as is shown by the overripened butter of Lot I. In determining what this deleterious factor is, it should be remembered that these changes took place at temperatures of 20 and even 40 degrees below the freezing point. We know that a comparatively high acidity was developed in the cream from which this butter was made, but there is also the possibility that other influences having little connection with the acid may have developed to affect the flavor of the butter.

The direct action of bacteria in causing changes at these temperatures is obviously excluded, but an indirect action may be found in the possibility that enzymes are secreted in the cream by bacteria and that these enzymes are able to act even at the low temperatures at which this butter was stored.

DETERMINATIONS OF BACTERIA IN THE BUTTER.

Qualitative and quantitative determinations of the bacteria in the cream and butter were made according to the following methods: Gelatin, and in some cases both gelatin and agar, plates were made of the cream at the various stages of the ripening. All media used contained 2 per cent lactose and were corrected to a reaction of +0.2 Fuller's scale, with the exception of plates made from Lot III, in which the reaction was +1.0. Anaerobic plates showed no bacteria not present on the ordinary aerobic plates. In making plates from the butter, samples of 5 to 10 grams each were taken, with proper precautions to prevent contamination, from five or six different places in each tub. The samples were melted at 40° C., and after thorough

mixing, 5 grams of each were weighed into flasks containing 500 c. c. of sterile water. These flasks were held in a water bath until the temperature reached 40° C. and were shaken vigorously to insure a thorough distribution of the bacteria. Plates with varying dilutions were made in the usual way.

The results of the bacterial determinations are given in Table 4 for the Lot I butter, in Table 5 for the Lot II butter, and in Table 6 for the Lot III butter.

TABLE 4.—*Bacteria in butter of Lot I.*

Method.	Initial number.	Storage temperature.	After six months' storage.	After nine months' storage.
° F.				
Unpasteurized cream:				
Churned sweet.....	8,983,000	-10 10 32	2,300,000 2,360,000 1,195,000	3,090,000 1,131,000 816,000
Starter added.....	2,893,000	-10 10 32	872,500 730,000 531,000	906,000 351,000 102,000
Normal ripening.....	3,924,000	-10 10 32	1,257,750 1,107,500 349,825	949,000 1,026,000 211,000
Overripened.....	9,825,000	-10 10 32	24,727,500 1,470,166 554,000	1,187,000 No butter. 124,000
Pasteurized cream:				
Churned sweet.....	386,166	-10 10 32	146,625 134,166 6,870,000	49,000 106,214 174,000
Starter added.....	2,800,000	-10 10 32	855,750 245,250 305,000	120,000 102,000 253,000
Normal ripening.....	945,000	-10 10 32	110,000 275,000 265,000	10,500 6,500 32,876
Overripened.....	1,956,000	-10 10 32	67,500 300,166 24,500	11,800 16,100 8,100

TABLE 5.—*Bacteria in cream and butter of Lot II.*

Method.	Bacteria per cubic centimeter in cream before churning.	Bacteria per gram in butter before storage.	Storage temperature.	Bacteria per gram in butter after storage.
° F.				
Not pasteurized:				
No starter.....	66,100,000	11,900,000	-10 10 32	490,000 365,000 153,000
Starter added.....	553,500,000	12,800,000	-10 10 32	726,000 133,000 64,000
Normal ripening.....	779,000,000	4,260,000	-10 10 32	376,000 297,000 106,000
Overripened.....	620,000,000	613,000	-10 10 32	13,000 5,000
Pasteurized:				
No starter.....	27,000	491,500	-10 10 32	28,000 90,000
Starter added.....	85,750,000	826,000	-10 10 32	 241,000 381,000
Normal ripening.....	781,000,000	13,650,000	-10 10 32	450,000 35,600 172,000
Overripened.....	880,000,000	38,400,000	-10 10 32	1,100,000 23,000

TABLE 6.—*Bacteria in cream and butter of Lot III.*

Method.	Bacteria per cubic centimeter in cream at time of churning.		Bacteria per gram in fresh butter.		Bacteria per gram in butter after storage at 10° F.	
	Total.	Liquefiers.	Total.	Liquefiers.	Total.	Liquefiers.
Unpasteurized:						
No starter.....	43,800,000	1,750,000	3,755,000	161,000	610,000	85,000
Starter added.....	226,000,000	540,000	7,233,000	94,000	563,000	1,000
Ripened.....	258,500,000	75,000	12,766,000	None.	469,500	None.
Overripened.....	362,500,000	40,000	3,136,000	None.	87,000	2,000
Pasteurized:						
No starter.....	195,500	13,300	88,500	20,000	1,344,000	56,000
Starter added.....	162,500,000	None.	10,300,000	3,000	2,375,000	1,500
Ripened.....	1,000,000,000	None.	11,520,000	None.	278,500	400
Overripened.....	526,000,000	None.	24,666,000	None.	420,000	None.

^a Development of *Oidium lactis* and *Bacterium lactis aerogenes*.

There is, of course, always the possibility that bacteriological methods do not show true bacteriological conditions. Assuming that the gelatin plates gave a correct indication of the nature and number of the bacteria present, there is little in the results in the Lot I butter to connect the bacteria in any direct way with the changes in the butter. The unpasteurized cream contained a total of 9,000,000 bacteria per cubic centimeter, of which 300,000 were of the liquefying type. There was an increase for a short time of the liquefying bacteria, but these were soon suppressed by the lactic-acid bacteria. When the maximum development was reached the lactic bacteria decreased slowly. There was, however, no apparent growth of any other kind of bacteria. The freshly pasteurized cream contained 1,192 bacteria. There was a small increase of nonlactic bacteria, but this was soon checked by the lactic bacteria, and the subsequent development agreed with that observed in the unpasteurized cream.

The cream from which Lot II was made contained 66,100,000 bacteria per cubic centimeter, which were reduced by pasteurization to 27,000. In the unpasteurized cream the lactic-acid bacteria soon gained the ascendancy and suppressed all other kinds. There was a considerable development of bacteria of the aerogenes type in the normally ripened pasteurized cream, but these were suppressed in the overripe cream by the lactic-acid bacteria. Otherwise the plates showed no bacteria which could be expected to influence the flavor.

The bacteria in the cream of Lot III followed the same general course. There was no appreciable growth of gas-forming bacteria in this cream, but in the overripened unpasteurized cream there was a small development of *Oidium lactis*. Ten days elapsed after this butter was taken from storage before it was received at the laboratory. During this time it was doubtless held at temperatures within the thermal growth limits of ordinary bacteria. There was a distinct increase of bacteria in the pasteurized sweet-cream butter in which

the two inhibiting factors, acid and salt, were low. This bacterial growth may have been responsible for the deterioration of this butter.

In all the butter stored at 10° and -10° F. there was a gradual decrease in the total bacteria. This was usually slightly more rapid at the higher temperature, but this difference in the rate of decrease was sometimes obscured by errors, due largely to the difficulty of securing a representative sample. At 32° F. this decrease was usually much more pronounced than at the lower temperatures. In several cases, however, there was an actual increase confined chiefly, if not entirely, to the torula group of yeasts. In one package the development was sufficient to make an actual increase in the total number of bacteria, which in the ordinary technique includes yeasts as well as bacteria. Usually the growth of yeasts was so much less than the decrease in bacteria that the total number showed a decrease. It has been demonstrated that some members of this group of yeasts may cause a decomposition of butterfat.^a

In some cases the change in the flavor of the butter stored at 32° F. might be accounted for by this development of yeasts, but in others in which there was an equal deterioration there was no appreciable increase of yeasts or bacteria.

The inference should not be drawn that the writers exclude the action of bacteria as a factor in causing changes taking place in butter. It is undoubtedly true that not only the flavor of the fresh butter but the change in flavor after the butter is made may be influenced by the bacterial growth in the cream, and under certain conditions bacteria and yeasts may grow in the butter itself. The uniform deterioration of the high-acid butter at the lower temperatures, however, could not be accounted for in this way.

POSSIBLE ACTION OF ENZYMES IN DETERIORATING HIGH-ACID BUTTER.

A plausible explanation of the changes may be found in the possible production of enzymes by the lactic-acid bacteria, which if carried into the butter would continue to act, as has been previously suggested, even at the lower temperatures at which it was stored. It is doubtful if enzymes are excreted by the lactic-acid bacteria, but it is well established that all cells contain enzymes which rapidly bring about the destruction of the cell after its death. Even the minute amounts of the decomposition products of the bacterial cells might affect the flavor of butter. Moreover, it is possible that enzymes not normally excreted are liberated by the natural death and disintegration of the cell. This is at least a possibility that can not be disregarded.

^a L. A. Rogers. "Studies upon the Keeping Qualities of Butter.—I. Canned Butter. United States Department of Agriculture, Bureau of Animal Industry, Bulletin 57.

The situation in the unpasteurized cream is complicated by the possible action of the enzymes of the milk, which include proteolytic, lipolytic, and oxidizing enzymes, but these enzymes would be destroyed by the pasteurization of the cream, which, as we have seen, did not prevent the deterioration.

If, as suggested, enzymes are liberated in any way by the lactic-acid bacteria, butter made from cream heated sufficiently after ripening to destroy the enzymes should not change at temperatures low enough to prevent the growth of bacteria and other organisms. In Table 7 are given the results of an experiment in which 10 per cent of starter was added to sour hand-separated cream, the mixture pasteurized at once at 180° F., cooled properly, and churned. One-half of this cream was pasteurized, starter added, and allowed to ripen over night.

TABLE 7.—Showing changes in butter made from cream pasteurized after ripening.

Method.	Score after one week (before storage).	Storage temperature.	Score after two months.	Score after five months.
		° F.		
a. Sour hand-separator cream, 10 per cent starter added, pasteurized at 180° F., cooled, and churned.	93	-10 10 32	83.7 84.7 82.7	84.2 84.0 85.0
b. Half of a. Pasteurized at 180° F., 10 per cent starter added, ripened over night, cooled, and churned.	92	-10 10 32	83.2 83.7 83.7	83.5 83.5 84.0

When these lots of butter went into storage one week after making, they scored 93 and 92, respectively. In two months both lots had developed, regardless of storage temperature, a rank, fishy flavor. All enzymes would be nearly if not quite destroyed by the temperature to which this cream was exposed. To assume that this marked change in flavor was caused by the development of bacteria would be contrary to our experience with other butter. These results have been duplicated many times in the course of other investigations.

One lot of butter was made in our experimental creamery in Albert Lea in the following way: The fresh cream was pasteurized at 170° F. To this was added 20 per cent of starter, and one-fourth was cooled and churned at once (designated as *a*). Another fourth was ripened overnight to an acidity of 0.41 per cent (*b*). The remainder was allowed to stand forty-eight hours. At this time the acidity was 0.59 per cent. Half of this remainder was properly cooled and churned (*c*), and the remaining fourth heated in a vat at 158° F. for ten minutes, after which it was cooled and churned (*d*). These butters were examined by numbers only when they went into storage one week after making, and again after four and one-half months of

storage at a temperature of 10° F. The results of the examination are given below:

TABLE 8.—*Changes in butter made from cream pasteurized after ripening.*

Portion and method.	Acidity.	Result after one week.	Score after four and one-half months storage at 10° F.
	<i>Per cent.</i>		
a. Starter added.....		Perfectly good.....	92. Tallowy.
b. Ripened.....	0.41	Very nice butter; good aroma.....	92. Tallowy. Slight storage.
c. Overripened.....	.59	Unclean.....	91. Stale-water flavor.
d. Overripened and heated.	.59	Extremely unclean.....	87. Old, tallow, rancid, grease.

It is apparent that the deleterious effect of high acidity was not due to any organism, enzyme, or other substance which can be destroyed by heat. It is evident, then, that some by-product of bacterial growth, unaffected by heat, had a marked influence on the flavor of the butter. It is probable that this was a by-product of the lactic-acid bacteria and that the by-product was lactic acid itself.

INFLUENCE OF LACTIC ACID ON FLAVOR OF BUTTER.

A number of experiments have been made to determine the influence of acidity developed in the cream by the addition of lactic acid. These have invariably given results similar to those shown in Table 9. In securing the results shown in this table one-third of a lot of pasteurized cream was cooled and churned at once (a). To the remainder were added small portions of chemically pure lactic acid until it showed an acidity of 0.36 per cent. Half of this was cooled and churned on the following morning (b). The acidity of the remaining third was increased to 0.41 per cent, cooled, held overnight, and churned the next day (c). The butter remained in the creamery refrigerator two weeks, when it was examined by numbers only, and shipped to storage at 10° F. It was examined again after three and a half months in storage with the following results:

TABLE 9.—*Influence of lactic acid on the flavor of butter.*

Portion and method.	Acidity.	Condition after two weeks.	Score after three and one-half months in storage at 10° F.
	<i>Per cent.</i>		
a. Churned sweet.....	0.099	Good.....	93.5. Sweet.
b. Lactic acid added.....	.360	Oily, unclean.....	89. Sweet, fruity.
c. Lactic acid added.....	.444	Oily, decomposed fat.....	87. Sweet, oily.

RESULT OF PRODUCING ACIDITY WITH OTHER ACIDS THAN LACTIC ACID.

Similar results have been obtained with butter made from cream acidified with acid other than lactic acid. These butters were made in the following manner: Sweet cream sufficient to make three churn-

ings was pasteurized in a continuous pasteurizer at 170° F. One-third was cooled and churned at once (*a*). To the remaining two-thirds acid was added slowly until the acidity expressed as lactic acid equaled about 0.22 per cent. This portion was divided and one-half cooled (*b*). The remaining portion was acidified to about 0.4 per cent and cooled (*c*). Portions *b* and *c* were churned on the following day. Lot 1 was acidified with lactic acid, lot 2 with acetic acid, and lot 3 with hydrochloric acid.

There was a slight development of bacteria in these creams, which was evidently checked by the higher acidity. The greater part of the bacteria present were of the lactic-acid type, or had no appreciable effect on milk. This butter was packed in sealed cans and a few days after making was shipped to storage and was examined by Mr. Credicott when about 15 days old. The results are given in Table 10.

TABLE 10.—*Showing the influence of different acids on the flavor of butter.*

Lot.	Acid added.	Acidity of cream.	Score after fifteen days.
		<i>Per cent.</i>	
1 a.....	None.....	0.144	88. Trifle unclean and very greasy.
1 b.....	Lactic.....	.216	88. Do.
1 c.....	do.....	.432	86. Fishy and greasy.
2 a.....	None.....	.126	90. Trifle oily, body weak and greasy.
2 b.....	Acetic.....	.216	87. Very greasy, and rancid on fishy order.
2 c.....	do.....	.350	84. Very fishy and greasy.
3 a.....	None.....	.126	90. Clean but greasy.
3 b.....	Hydrochloric.....	.225	90. Trifle unclean and oily.
3 c.....	do.....	.450	84. Very fishy, greasy.

This table shows that this action is not peculiar to lactic acid or even to organic acids.

It would appear, therefore, that the acidity of the cream has a direct influence on the changes in the butter. This is, of course, usually complicated by the influence of other factors, many of which are still undetermined. Just what action the acid has and what parts of the butter are changed to bring about the undesired flavors we are not prepared to say at this time, but investigations are in progress which it is hoped will throw some light on the complex chemical changes controlling the flavor of butter.

THE MANUFACTURE OF COMMERCIAL BUTTER FROM SWEET CREAM.

To the person interested in the application of these results to practice it is obvious that butter which market conditions require to be held for any length of time should be made with as little acid as possible. This is especially true of butter held for several months in cold storage and butter canned for use on shipboard or for export to tropical countries. While the object of this investigation was to determine the causes of change in butter, the results allow some com-

parisons to be made of the commercial value of the different methods of butter making. The deterioration in storage of butter made from overripened cream is well known, and this investigation has shown clearly that butter made from cream ripened according to the usual creamery practice changes much more than butter made with a low acidity. A number of trials were made to determine if adding sufficient starter to cream to improve the flavor of the butter would seriously impair the keeping quality.

The keeping quality of butter made from sweet pasteurized cream is compared in the next table with that of butter made with starter. The scores given in this table are the average scores of six lots of butter made in the creamery at Bloomer, Wis. The cream from which this butter was made was pasteurized in a Farrington pasteurizer at temperatures varying from 155° to 175° F. The lot was divided, a large starter added to one-half, and each portion cooled and churned at once. In a few cases one-half was ripened, but this butter was so much poorer than the sweet-cream butter that it was not stored. The scorers were Messrs. Kieffer, Smarzo, Credicott, and White:

TABLE 11.—*Showing relative keeping quality of butter made from pasteurized sweet cream, sweet cream with starter added, and normally ripened cream.*

Lot and method.	Storage temperature.	Average score after four months.	Average score after eight months.
	° F.		
1 a. Sweet cream.....	-10	92.6	93.0
	10	91.3	93.1
	32	89.3	91.0
1 b. Starter added.....	-10	91.0	92.5
	10	91.3	92.3
	32	89.3	89.0
2 a. Sweet cream.....	-10	90.3	92.6
	10	90.8	92.6
	32	90.6	88.6
2 b. Starter added.....	-10	92.2	94.3
	10	92.1	93.5
	32	90.0	92.0
3 a. Sweet cream.....	-10	90.6	92.1
	10	90.6	91.8
	32	89.2	88.3
3 c. Ripened normally.....	-10	90.4	89.6
	10	88.5	88.6
	32	85.7	83.3
4 a. Sweet cream.....	-10	91.8	92.7
	10	91.4	92.7
	32	92.0	93.0
4 b. Starter added.....	-10	91.9	93.0
	10	91.2	92.8
	32	87.3	87.2
5 a. Sweet cream.....	-10	91.1	93.0
	10	91.5	93.0
	32	92.2	91.5
5 b. Starter added.....	-10	92.1	93.0
	10	90.9	92.1
	32	88.0	86.0
6 a. Sweet cream.....	-10	91.3	93.5
	10	90.6	91.5
	32	89.6	92.2
6 b. Starter added.....	-10	91.0	93.0
	10	90.8	92.7
	32	87.3	89.3

The foregoing table is summarized in Table 12, which gives the average of the scores of the six lots of sweet-cream butter and of the five lots of butter made from sweet cream with starter:

TABLE 12.—*Showing average scores of butter made from pasteurized sweet cream and sweet cream with starter added.*

Method.	Storage temperature.	Average score after four months.	Average score after eight months.
	° F.		
Sweet cream (six lots)	-10	91.28	92.81
	10	91.03	92.45
	32	90.32	90.73
Starter added (five lots)	-10	91.64	93.16
	10	91.26	92.68
	32	88.38	88.72

The foregoing table shows that at the two lower temperatures there was little or no advantage in the sweet-cream butter over that with the starter, but at 32° F. there was a decided difference in favor of the sweet-cream butter. Butter has been made under commercial conditions by this method in large quantities, and when sold after several months in storage was considered highly satisfactory.

In the work described in the foregoing pages we have given little attention to sweet-cream butter for immediate consumption. We have, however, a number of scores of fresh butter, most of which are comparisons of butter made from pasteurized sweet cream with and without starter added.

In three lots sent to the New York market the butter made from cream with starter added was scored from 1 to 2½ points higher than the sweet-cream butter. On the other hand, in eight lots of butter sent to the Fox River Butter Company the average score of the sweet-cream butter was 93.7, against 93.3 for the butter with starter. The difference in favor of the sweet-cream butter was greater in the butter eight or ten days old at the time of scoring, while in the butter scored immediately after making the highest score was given to the butter with starter. In four lots in which sweet-cream butter was compared with butter made from cream ripened in the normal manner, the former received an average score of 94 against 91.2 for the ripened-cream butter.

The difference between the scores given the butter sent to New York and that sent to the Fox River Butter Company may be explained by the demand of the market and the personal tastes of the scorers. The New York market calls for a butter with a decided flavor, and the mild sweet-cream butter consequently received a low score. The scorer at the Fox River Butter Company, on the other hand, gives a good score to a butter free from any objectionable flavors. The scores of these lots are given in Table 13:

TABLE 13.—*Scores of fresh butter made from pasteurized sweet cream, sweet cream with starter added, and cream ripened normally.*

Lot and method.		New York (Kieffer) score.	Fox River Butter Com- pany score.
1	a. Sweet cream.....		94
	b. Starter added.....		94
2	a. Sweet cream.....	92.5	94
	b. Starter added.....	93.5	92
	a. Sweet cream.....	92	94
3	b. Starter added.....	94	92.5
	c. Ripened.....		90
	a. Sweet cream.....		94
4	b. Starter added.....		93.5
	a. Sweet cream.....		93
5	b. Starter added.....		94.5
	a. Sweet cream.....		93
6	b. Starter added.....		94
	a. Sweet cream.....		94.5
7	b. Starter added.....		94
	a. Sweet cream.....	90.5	93.5
8	b. Starter added.....	93	92.5
	a. Sweet cream.....		94.5
9	b. Ripened.....		92
	a. Sweet cream.....		94
10	b. Ripened.....		92
	a. Sweet cream.....		93.5
11	b. Ripened.....		91

These results show at least that butter may be made from sweet cream which will after long storage at comparatively high temperatures be classed as good butter by competent judges. The method for making this butter is so simple that a uniform output with little danger from undesirable fermentations can be insured. The trouble and expense of starters are eliminated. In churning sweet cream it is essential that careful attention be given to the churning temperature and to the speed of the churn, otherwise there is likely to be an unnecessary loss of fat in the buttermilk. Care should be taken in storing butter made from sweet cream to guard against woody or other extraneous flavors, which are likely to be more noticeable in this mild-flavored butter than in butter with a high flavor.

The Dairy Division is not advising the adoption of this method for general use, but its trial for butter intended for storage is recommended.

CONCLUSIONS.

Butter frequently undergoes marked changes, even when stored at very low temperatures.

These changes are more marked as the acidity of the cream from which the butter is made is increased.

No bacteria were found in the cream or the butter which could reasonably be expected to be the cause of the more rapid deterioration of the high-acid butter.

The changes in the high-acid butter were not checked by heating the ripened cream, which shows that they were not brought about by enzymes secreted with or in the cream and carried into the butter.

Marked changes of an undesirable nature were produced in butter by acidifying pasteurized cream with various acids. These changes did not take place all at once, but were of a progressive nature.

The results indicate that the acid developed normally in the cream by the action of the lactic-acid bacteria, or added directly to the cream in the form of pure acid, brings about or assists in bringing about a slow decomposition of one or more of the labile compounds of which butter is largely composed.

Butter can be made commercially from sweet pasteurized cream without the addition of a starter. Fresh butter made in this way has a flavor too mild to suit the average dealer, but it changes less in storage than butter made by the ordinary method, and can be sold after storage as high-grade butter.

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Issued October 5, 1909.

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BUREAU OF ANIMAL INDUSTRY—BULLETIN 115.

A. D. MELVIN, CHIEF OF BUREAU.

CAMEMBERT CHEESE PROBLEMS IN THE
UNITED STATES.

BY

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LETTER OF TRANSMITTAL.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY,
Washington, D. C., July 8, 1909.

SIR: I have the honor to transmit herewith, and to recommend for publication in the bulletin series of this Bureau, a manuscript entitled "Camembert Cheese Problems in the United States," by Dr. Charles Thom, mycologist in cheese investigations of the Dairy Division of this Bureau. The paper describes the latest phase of the cooperative work in connection with European varieties of soft cheese which has been in progress for the past five years between the Dairy Division and the Storrs (Conn.) Agricultural Experiment Station.

The greater part of the work mentioned has related to the manufacture of the Camembert type of cheese in the United States, this being a well-known variety and one that has already been produced on a commercial scale with varying success in some of our Northeastern States, besides being extensively imported. Efforts to establish the industry in the United States have not been wholly successful, and it appears from Doctor Thom's investigations that this is due to the fact that climatic conditions are unfavorable during the greater part of the year in most of the regions where factories have been located. Of a number of American cities studied, San Francisco alone was found to have climatic conditions approaching closely those of the Camembert district of France. It is believed, however, that the climatic disadvantages in sections where they exist can be overcome by constructing factories in such a manner as to provide proper control of temperature, humidity, and ventilation.

Doctor Thom has made a very thorough investigation of the peculiar problems incident to the manufacture of Camembert in this country, and it is hoped that the results of his work, herein described, will be of value and assistance in establishing the industry upon a more successful and permanent basis.

A list of the publications previously issued by this Bureau upon the subject of Camembert cheese will be found at the end of the bulletin.

Respectfully,

A. D. MELVIN,
Chief of Bureau.

Hon. JAMES WILSON,
Secretary of Agriculture.



CONTENTS.

	Page
Summary.....	7
Introduction.....	10
Factory equipment.....	10
The receiving room.....	11
The cheesemaking room.....	11
Equipment of cheesemaking room.....	11
Conditions required.....	14
Standards of composition of cheese and milk.....	15
Cheese.....	15
Standard milk for Camembert.....	16
Relation of fat and water to texture.....	17
Starters and acidity.....	18
The prevalence of gassy curd at certain seasons.....	19
Amount of starter necessary for ripening milk.....	21
Details of cheesemaking.....	22
Inoculation with Camembert mold.....	26
The ripening of Camembert cheese.....	27
Equipment of ripening rooms.....	30
The newly made cheese.....	31
The ripening agents.....	32
Oidium ripening.....	34
Other organisms occurring on Camembert cheese.....	35
Conditions of ripening.....	36
Percentage of water.....	36
Temperature.....	36
Relative humidity.....	37
Other conditions.....	38
The climatic factor.....	39
Comparison of American and French climatic conditions.....	40
Construction of rooms for cheese ripening.....	45
Stages of ripening.....	46
When to market the cheese.....	49
The American market.....	50
Shall the factory make Camembert only?.....	50
The cooking of Camembert cheese.....	51
Making Camembert cheese on the farm.....	51
Acknowledgments.....	53
Publications relating to Camembert cheese.....	53

ILLUSTRATIONS.

	Page.
FIG. 1. Camembert cheesemaking room in American factory	12
2. Camembert cheese factory at Lisieux, France	27
3. "Halloir" or first ripening room in American Camembert factory ...	28
4. "Sechoir," second, or drying room in American factory	29
5. Another part of the French factory shown in figure 2.....	30
6. Another French factory, showing large windows, with blinds, in "sechoir"	31

CAMEMBERT CHEESE PROBLEMS IN THE UNITED STATES.

SUMMARY.

The following summary presents the main features of the processes and problems involved in the manufacture of Camembert cheese and gives references to pages where these subjects are discussed more in detail.

EQUIPMENT AND CONDITIONS.

The descriptions of equipment and conditions desired in making Camembert cheese are based upon the practice of the factories in France and in the United States. Pages 10-15.

MAKING PROCESS.

Making-room temperature, 60 to 75° F., about 68° preferred; keep temperature as uniform as possible. Page 14.

Air of room, moist to wet. Page 14.

Milk, whole or very slightly skimmed (0.5 per cent), not over eighteen hours old. Pages 16-17.

Starter, any standard form, 0.5 to 1 per cent, twelve to eighteen hours ripening (overnight) below 57° F. Pages 18-21.

Acidity at renneting, 0.20 to 0.23 per cent (phenolphthalein). Pages 18-21.

Temperature of renneting, 86° F., limits, 84 to 90° F.

Rennet, 3 to 5 ounces per 1,000 pounds (10 to 15 cubic centimeters per 100 pounds). Page 22.

Curdling time, one and one-fourth to one and one-half hours or longer. Page 22.

Cutting curd, not advised.

Dipping, uniform distribution of curd into hoops. Page 23.

Draining, till next morning without turning.

Inoculation with molds, not necessary except when establishing factories. Page 26.

Turning, early second morning.

Salting, when firm enough to handle, usually eighteen to twenty-four hours after dipping. Several forms of manipulation used. Page 24.

Draining after salting, in making room, twenty-four to forty-eight hours. Page 25.

Cheese ready for ripening, contains 57 to 60 per cent water; when fully ripe, 47 to 50 per cent water. Cheese outside such limits calls for extra care in handling. Pages 31-32.

RIPENING PROCESS.

Conditions in ripening rooms.—(1) Temperature, 52 to 58° F. recommended. Lower temperature lengthens the process; higher temperature shortens it and hastens decay. Page 36.

(2) Relative humidity, limits about 83 to 90 or 92 per cent. Optimum depends upon temperature selected and water content of cheeses. For cheeses evenly drained and fairly firm at beginning of ripening (perhaps 57 to 59 per cent water) probably the optimum would be 86 to 88 per cent relative humidity at 52 to 54° F. Pages 37-38.

First two weeks.—Cheeses are kept upon coarse matting (clayons). Conditions should be controlled to produce a moderately thin rind showing well distributed but not heavy areas of mold interspersed with patches beginning to show reddish slime. Relative humidity must be held high enough to permit the slime to begin to show with the mold, but not so high as to prevent the appearance of the mold. Cheeses will lose probably about 3 to 6 per cent of water in this time, according to handling. Traces of softening under the rind show at the end of two weeks. Cheeses must then be removed to smooth boards or wrapped and boxed. Pages 46-47.

Third week.—Slime areas increase without other changes in appearance. Softening progresses rapidly. The rate of change depends on the temperature and the percentage of water still present. Enough evaporation must be allowed to bring the softened protein to the consistency desired (commonly 50 to 51 per cent of water at the end of the time). The progress of this change can be constantly determined by feeling the cheeses. Ripening of the proper texture and flavor must be well begun and the water content lowered to a safe percentage before cheeses can leave the factory with assurance of success in their further handling. Page 47.

Further handling.—According to the market demand, cheeses may be boxed and their further ripening completed in the cellars or in storage in the dealers' hands. The ripening should not be complete before the end of the fourth week and may often desirably be lengthened considerably beyond that time. The progress sought can be controlled to a large degree by controlling the temperature of the storage room, or ripening cellar, if one is used. If cheeses have the proper consistency at the end of the third week, proper care alone should assure good results in the further ripening. This responsibility falls upon the dealer or consumer. Pages 48-49.

Gassy curd.—The making process of Camembert subjects it to greater risks from the development of gassy curd than most other cheeses. During three successive years this trouble has been much greater during December, January, February, and March than the rest of the season. A seasonal prevalence of the gas-producing acid organism, *Bacillus lactis aerogenes*, is indicated for these months or parts of them. The introduction of 0.5 per cent, or slightly more, pure starter with ripening over night at 50 to 57° F. has produced sufficient ripening to reduce gas formation to a minimum, without raising the acidity test (phenolphthalein) above 0.22 to 0.23 per cent. Pages 19-21.

Changes in composition during ripening.—The changes in composition during the ripening period are the neutralization or destruction of acidity, the softening of the cheese due to proteolysis of the casein, and the lowering of the water content about 10 per cent. The fat is little affected. The activity of the ripening agents, and consequently the rate of ripening, is found to be closely dependent upon the amount of excess of water in the fresh cheese above the water content in the ripe cheese and to the rate and conditions under which that water is evaporated. Pages 32-34.

Temperature and humidity.—The temperature and relative humidity of the air in the ripening rooms determine the rate of loss of water in the ripening of the cheese. The temperature limits recommended are 52 to 58° F. The relative humidity indicated varied from 84 to 90 per cent, or even higher.

COMPOSITION OF CAMEMBERT CHEESE.

Analyses of imported and domestic Camembert have been tabulated and discussed, to determine as closely as possible a satisfactory standard for this variety of cheese. Marked variations have been found in various brands. The analyses of cheeses selected as representing choice conditions of texture and flavor place the average of the best cheeses within approximate limits as follows: Water, 47 to 50 per cent; fat, 25 to 28 per cent; protein, 18 to 21 per cent. Variations outside these limits are common, but seem to involve more risks of defects than those within these limits. Pages 15-16.

CLIMATIC DIFFICULTIES IN THE UNITED STATES.

Tabulation and comparison of mean temperatures and mean relative humidities for cities in northern France and certain American cities in dairy regions show that in America mean temperatures are either too high or too low for Camembert cheese ripening, except during portions of September, October, and November. In the same cities the mean relative humidity remains too low in nearly every month of the year for ripening this kind of cheese. San Francisco alone of the American cities studied shows conditions closely similar to those in northern France. Pages 39-44.

Ripening rooms built for climatic conditions found in France have failed to give success in America. Either domestic manufacture of Camembert must be abandoned in most sections or the construction of the rooms for cheese ripening must be so modified as to obtain the conditions desired. Pages 27-31.

Factories to succeed in the Eastern States must provide control of temperature and relative humidity within closer limits than those obtainable with the French plans hitherto used. This may be obtained by better insulation of the rooms already built or by the construction of new rooms which may be partly or entirely below the surface of the ground. In either case the building must provide means for thorough but controlled ventilation sufficient to carry off moisture as fast as required. Pages 45-46.

FACTORY AND FARM PRODUCTION.

Camembert cheesemaking for the general market is a factory proposition in which production upon a large scale conduces to economy of labor and uniformity of results. A good grade of Camembert can be made and ripened upon the farm with comparatively simple and inexpensive equipment. The difficulty of making uniform cheeses is greater when working upon a small scale. Such cheesemaking can not at present be advised except for home use or for sale to a special market served directly by the producer. Pages 50-53.

INTRODUCTION.

Numerous practical and scientific problems have been encountered in attempting to establish the manufacture of Camembert cheese in the United States. Some of these, together with the practices followed in the first two years of the investigation, have been discussed in previous papers. Before and during the progress of this early work several factories undertook to produce this cheese in America. Most of these companies established plants with the object of reproducing as nearly as possible the buildings and equipment successful in France, and employed experienced cheesemakers from that country to carry on the work. At one time the product of these factories made up fully one-fourth the total amount of Camembert consumed in America. The production and sale of this cheese was, however, attended by uncertainties as to market and by numerous losses in the factory. Some of these difficulties were readily recognized, but in many cases even the experienced maker failed without being able to find the reasons for his losses. So much difficulty and discouragement have attended these enterprises that some of them have been entirely abandoned, and the product from all has been greatly reduced.

In continuing the investigation, experimental work at Storrs has been supplemented by a study of the problems of the market (see Circular 145, Bureau of Animal Industry), and by visits to all the factories carrying on this work.

This paper is a report of progress upon the studies of the problems which have come up in this work, together with modifications of handling that will bring the processes recommended into substantial harmony with factory practice.

In spite of the failures which have occurred in the past, there is good reason to believe that a readjustment of methods to conditions will eventually bring permanent success. Without such readjustment, the transplanting of the Camembert industry to our Eastern States has so far disappointed the investors. It can not, however, be claimed that all the difficulties have been overcome in the practices discussed in this paper, but much progress has been made toward practical working success and toward correct interpretation of the causes for past failures and losses.

FACTORY EQUIPMENT.

The equipment of a Camembert cheese factory comprises most of the apparatus and utensils common to all creamery work. A room for receiving and weighing the milk, apparatus for testing the amount of fat in milk, steam for heating and sterilizing purposes, etc., such as are found in any up-to-date dairy, are essential. In other words, all the usual contrivances that facilitate the rapid and sanitary handling of milk in large quantities must be provided.

All of the rooms in the factory should be constructed with a view to the maintenance of strict cleanliness. In the room in which the milk is delivered and also in the cheesemaking room the floors must be flushed daily with water to remove any milk or curd that otherwise would form a breeding place for germs or insects. To this end the floors should be of cement and should slope toward one or more drainpipes. The walls also should be of some material that can be kept clean, and should be painted or whitewashed from time to time. The cheesemaking room, as well as all the other rooms where the cheese is handled, must be protected from flies and other insects. Unless great precautions are taken, swarms of flies will invade the room and deposit their eggs on the cheese, and a few days later, in the ripening room, these eggs will hatch out into maggots. All the windows must therefore be screened with wire netting of fine mesh, and every effort made to prevent the entrance of flies when the doors are opened.

THE RECEIVING ROOM.

The room for receiving the milk requires the same equipment as in ordinary dairy work. A vat with capacity sufficient for the mixing, heating, and ripening of all the milk used each day is usually provided. There may be either a single vat or several, but the use of the larger vat insures the thorough mixing of the whole milk supply, and hence a more uniform composition of the whole lot from day to day than would be obtained from the use of several vats. Aside from the desirability of mixing the milk for uniformity, a small receiving vat from which the milk runs directly through a milk heater to the curdling cans is equally efficient.

THE CHEESEMAKING ROOM.

The floor of a cheesemaking room is usually cement, either upon or slightly below the surface of the ground. The room should be well lighted. Double sashes are used to protect the room from abrupt changes of temperature, and usually two or more of the windows are well screened and arranged to open conveniently for ventilation when desired. To avoid the accumulation of dirt the inside sash should be hinged and close up flush with the wall surface, leaving no ledges to gather filth.

Figure 1 illustrates the cheesemaking room and its equipment in a Camembert factory in the United States.

EQUIPMENT OF CHEESEMAKING ROOM.

Aside from the general equipment for all creamery work, such as steam, water, and hose, the special equipment and apparatus for Camembert cheesemaking will be briefly described.

Tables.—Enough table surface is demanded to accommodate the cheeses to be made in two days. These tables ("tables à mouler") are 36 to 42 inches wide, fastened in pairs to the opposite sides of two vertical pillars about 32 inches above the floor. A raised edge on each side prevents whey from running from the table to the floor. The tables slope toward the pillars to which they are fastened and toward one end, so that a single gutter connecting their ends carries off the whey from all the tables. They are constructed of wood with smooth surface, sometimes of wood covered with galvanized iron. It is best not to have the wood protected by metal or any heat-conducting material. The temperature of the curd being several degrees



FIG. 1.—Camembert cheesemaking room in American factory, showing arrangement of tables, aisles, curdling cans, and milk-distributing pipes.

above that of the room, the curd cools more or less rapidly while draining, and the rate of loss of heat is increased when the curd is in contact with a cold sheet of metal. This retards draining and produces an uneven texture on account of the different rate of cooling of the top and bottom of the cheese.

In most factories, directly above the draining tables and fastened to the pillars in the same way, 3 feet above the tables, are shelves of about the width of the draining table. They are used for draining the cheese during the second and third days, when space is needed. These shelves are not used except when space is urgently needed, and often not at all.

Aisles.—The aisles between the tables should be wide enough for the maker to work comfortably. Sometimes only 26 inches are allowed, but 32 to 36 inches would be more comfortable working room.

Draining mats.—These are imported from France. The matting is made in strips like cloth of different widths and is bought by the roll. It is composed of delicate wood strips held together by thread. The matting purchased should be exactly as wide as the draining tables. It is cut into lengths convenient for handling and washing. These may be the full length of the tables unless the tables are very long.

Hoops or forms.—The number of hoops provided should be twice the number of cheeses to be made each day. The hoops used vary slightly (perhaps a quarter of an inch) in diameter in different factories. They are preferably made of heavy tin with edges turned and soldered. The hoops used in factories visited have been 5 inches high and $4\frac{1}{2}$ inches in diameter. The diameter used by different makers often differs one-eighth of an inch from this average. Each hoop is perforated with three rows of holes one-twelfth of an inch in diameter and about 2 inches apart in the row. Although hoops 5 inches high are regularly used, it is often necessary to fill them up after the curd has been dipped some time. When this is found to be the case, it might be desirable to make the hoops half an inch higher. Some have used also a low hoop for draining on the second day. Its use is not general and is not recommended.

Disks.—In some factories heavy tin disks are provided. These exactly fit the hoops and are used as weights to produce a smooth upper surface upon the cheeses. These have not been used much in this country and did not give satisfaction when tried in our work. In some factories a handle carrying a rubber sucking disk is provided to remove these disks.

Dippers.—The curd is transferred from curdling cans to hoops by means of long-handled dippers which are small enough for the bowl of the dipper to be lowered into the hoops.

Curdling cans.—Curdling cans (shown in fig. 1) are made to hold about 200 pounds of milk each. These cans are made of heavy metal and taper from about 12 inches in diameter at the bottom to 20 to 24 inches at the top. Handles at the top make them more convenient to move.

Trucks.—For each one or two makers dipping cheese a truck must be provided. This consists essentially of a round base with a rim perhaps half an inch high, into which the curdling cans fit readily. Under this base rollers provide easy motion in any desired direction. The height of the truck plus the height of the curdling can should bring the edge of the can very nearly to the top of the hoops when they are arranged upon the draining table. This will minimize the labor of dipping as described later. The trucks are shown in figure 1.

Curdling shelf.—A shelf conveniently placed should be just high enough from the floor to allow the curdling cans to slide readily upon the trucks. The tables are usually placed with one end toward the windows, the aisles between and across the inner end, with curdling cans arranged on their shelf along the inner wall of the room. Arrangement is, however, a matter of convenience. Instead of a wooden shelf, sections of the concrete floor along the wall are often simply raised above the main level of the floor high enough to move the cans easily to the trucks.

Salting boards.—Boards or trays are provided for handling freshly salted cheeses while they remain in the making room. These are made from $\frac{3}{4}$ -inch smooth matched lumber, held together by cleats to make the boards or trays about 24 by 30 inches—large enough to carry 30 cheeses.

Other apparatus has sometimes been used or recommended for the cheesemaking room. Vats, for example, can be used instead of curdling cans, but they entail a larger initial cost, to which must be added the constant extra labor of lifting curd by dipperfuls from the vat across the aisle to the hoops. This extra labor is in itself prohibitive of the use of vats. The use of corrugated draining boards upon the tables is added labor and expense, without compensating advantage. The apparatus described here has shown its economy by its general acceptance in cheese factories.

CONDITIONS REQUIRED.

Temperature.—The limits are 60° to 75° F., the preferred temperature being 68° F. These limits are recommended by students of French practice, and experimental work has given the same results. If the temperature is allowed to go below 60° F. the drainage of the cheese is delayed or even entirely interrupted. If the room is too warm the danger of developing gassy cheeses is much greater. Bacteriological studies by Conn, Esten, Stocking, and others have fixed 70° F. as a condition under which the typical lactic bacteria work more rapidly than gas-producing types; whereas from 70° to 98°—as the temperature approaches blood heat—the gas-producing species gradually increase in activity until they reach dominance. At the high temperatures the rate of separation of whey increases also. It has been found desirable to hold the making room slightly under rather than over 70° F., if there is a preference within the limits given.

Humidity.—The room should be kept moist—not fully saturated, but so moist that surface evaporation is slow. Rapid evaporation causes the curd to adhere to the hoops and makes badly shaped cheeses. Cheeses tend to dry out and shrink quickly in dry air. The upper face of a cheese will shrink in diameter often half an inch while draining in a dry room. Rapid evaporation produces a thin,

hard rind over wet curd instead of a gradual and uniform reduction of water content throughout the whole cheese. To obtain the proper water content a cheese must drain out, not dry out.

Ventilation.—The actual rate of ventilation in the making room appears to matter very little so long as the air is kept pure. In our climate the changes of temperature are more rapid and the air averages much dryer than in Normandy. Ventilation must, therefore, be much more closely restricted to avoid dryness than in French factories. The cheesemaking area in the United States is also much higher above sea level, with prevailing winds blowing over large land areas. All these things contribute to the need of greater restriction of ventilation in the making room than appears in French factories.

Milk.—Good, clean milk, not over eighteen to twenty hours old, forms the best basis for work. Milk older than this, if well cooled and cared for, may be used; but the danger of gassy fermentations increases so greatly that our experience amply justifies the French practice of using the fresh milk of the morning mixed with the well cooled milk of the night before.

STANDARDS OF COMPOSITION OF CHEESE AND MILK.

CHEESE.

The practice of removing a small part of the fat from the milk used in making Camembert cheese is admitted to be very common in France. The amount of such skimming varies with the maker, the richness of the milk, and the season. The several brands of this cheese appearing in the American market have been purchased and analyzed from time to time during the past five years, to establish, if possible, standards for comparison. The following table presents fairly representative analyses for cheese appearing upon the market in fall and winter:

TABLE 1.—*Analyses of Camembert cheese in American markets.*

Sample No.	Analyst.	Water.	Fat.	Protein.	Proportion of fat to protein.
		<i>Per cent.</i>	<i>Per cent.</i>	<i>Per cent.</i>	
1	Dox.....	47.50	26.30	21.80	1:0.82
2	do.....	45.59	27.71	21.40	1: .77
3	do.....	46.36	27.78	21.21	1: .76
4	Edmond.....	48.41	27.01	19.36	1: .71
5	do.....	48.79	26.72	18.75	1: .71
6	do.....	43.08	32.13	21.27	1: .66
7	do.....	44.25	31.09	19.69	1: .60
8	do.....	50.59	26.30	18.83	1: .71
9	Dox.....	47.03	26.67	20.32	1: .73
10	Edmond.....	54.41	23.04	16.83	1: .73
11	do.....	51.23	25.68	17.61	1: .68
12	do.....	47.69	27.32	19.05	1: .69
	Average of 12 analyses.....	47.91	27.33	19.66	1: .71

Cheese No. 4 in the table reached the analyst in almost perfect condition, both as to texture and flavor; it may be regarded as giving nearly a typical analysis, therefore, for the best Camembert. Nos. 8 and 9 were made in America; all of the others were imported.

Taking the relation of fat and casein given by Van Slyke,^a cheeses Nos. 6 and 7 represent approximately milk testing 4 per cent fat. This allows a casein loss of 0.1 per cent and a fat loss of 0.2 to 0.25 per cent. Inspection of the ratio of fat to protein in every other case shows evidence of the removal of a small part of the fat. Some of these cheeses were manifestly low in water content from exposure in the market and consequent evaporation. The same is very evidently true of certain of the analyses given by Von Klenze and by Doane and Lawson.^b

The percentage of water as shown in this table was from 43+ to 50+, but in those cheeses analyzed in the best condition the percentage has been found to vary from 46 to 50, or slightly more; probably 48 per cent, with a variation of 2 per cent either way, would include most of the better cheeses as they reach the American market. It must be admitted, however, that very many good cheeses found in the open market show signs of shrinkage, which indicates that they had contained 52 to 55 per cent of water, or even more, at the time of shipment. Fifty per cent may therefore be considered a fair average for partially ripe cheeses when they go to market. The further ripening commonly reduces this 2 and often 3 to 4 per cent without serious loss of quality.

In fat content all the cheeses in the table excepting three (Nos. 6, 7, and 10) varied little more than 2 per cent—that is, from 26 to 28 per cent. Nos. 6 and 7 were especially high in fat and correspondingly low in water, and appeared to be the output of the same factory. The column marked “Protein” shows almost as narrow a variation on the average cheese, running from 19 to 21 per cent. Analysis of the richer grades of American milk indicate that at least 0.5 per cent of fat may be removed from milk testing 4.25 per cent or over, and still leave the ratio of fat to protein as high as found in good imported cheeses. In very rich milk the fat figure would yet be relatively too high in many cases.

STANDARD MILK FOR CAMEMBERT.

The analyses given, supplemented by those of cheeses made from milk standardized at from 3 to 6 per cent fat content, show that

^a Van Slyke, L. L., and Publow, C. A. *The Science and Practice of Cheese-making*, p. 233.

^b Doane, C. F., and Lawson, H. W. *Varieties of Cheese*. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 105.

Camembert cheese requires good milk, but not milk excessively rich in fat. The closest correspondence with the composition of the best imported cheese was obtained by using Jersey milk standardized to 3.8 per cent fat after the removal of 0.5 per cent of the fat—a percentage still rather above that of average factory milk. The ratio of fat to protein in the average analysis (1:0.71) indicates the removal of some fat^a as the usual condition. Although certain factories manifestly use whole milk, in most of them a moderate skimming is always practiced. There appears to be good reason to believe, as claimed by Roger, that the ratio of fat to protein shown by the average analysis of Camembert produces a cheese of better texture and more satisfactory flavor than when the fat is greatly increased, as is the case with unmixed Jersey milk, for example.

Since, however, there is no profit in skimming, it should be practiced as little as possible. For profitable results cheeses should be made to carry all the fat they will without causing injury to texture and flavor. If we calculate Camembert at approximately 50 per cent water, every pound of milk solids (fat and casein) carries with it an equal amount of water in making up the yield of cheese. Milk fat, therefore, sells in the same markets more profitably as Camembert cheese than as butter. The removal of more than 0.5 per cent of fat is probably not warranted with ordinary factory milk. In some cases a less amount would be better, but some fat should ordinarily be removed from all milk to obtain the proper proportions of fat and casein in this cheese.

RELATION OF FAT AND WATER TO TEXTURE.

In studies of cheeses made from the same milk standardized to high and low fat content it is uniformly found that under conditions otherwise the same the milk low in fat produces cheeses higher in water content. In Camembert, therefore, as in the hard cheeses, to obtain the same texture from milk partly skimmed as from rich whole milk the loss of fat is partly, at least, replaced by higher percentages of water.

^a Van Slyke and Publow (loc. cit., p. 165) give the ratio of fat to casein in the milk of different breeds as varying from 1:0.67 for Holstein-Friesian to 1:0.52 for the highest grade of Jersey milk. In no case do they find factory milk to give a higher ratio than 1:0.67 or 0.68. The experiments here cited were made with milk originally high in fat. The figures given by these authors have been confirmed by work done at the Storrs station and by unpublished figures collected by the Dairy Division from a large number of factories.

STARTERS AND ACIDITY.

Some makers prefer not to use starters. Our experience has always justified the use of starters, the result being a reduction of losses from gas and the production of a better draining cheese.

The so-called "natural" starters, when entirely free from gas or taint, are always good. Many factories use no other kind. Butter-milk when in good condition produces acidity rapidly and gives a product uniform in flavor and texture. It is subject to rapid deterioration, however, and if used must be watched carefully for gassy fermentation.

The commercial starters as a rule produce excellent final acidity and seem to result in cheeses of stronger flavor. It has been generally more difficult, nevertheless, to obtain uniform results with them. Apparently starters made from these cultures have varied considerably in the rate and intensity of their activity; hence cheeses made from day to day differed more than those made with a natural starter. With proper care commercial starters probably give the best results on an average.

Acidity at renneting time.^a—From 0.20 to 0.23 per cent has given our best results. If the acidity is higher than 0.25 the curd is not so smooth—that is, when broken between the fingers it feels grainy or mealy, "rough." If acid is too low—0.16 to 0.18 per cent—it requires more rennet, longer curdling time, and longer draining time, but produces a good curd in our experiments. Since, however, milk usually enters the factory with some acidity, it is hardly practicable to plan to work at an acidity lower than 0.20 per cent. The practice has therefore been adjusted to such milk as is usually obtainable. On the same grounds, with equal reason, milk with higher acidity than 0.20 per cent when received is not desirable, and cleaner and fresher milk should be insisted upon. Milk with excessive acidity when received quite often develops gas or other fermentation (yeast, bad odors, etc.), and this indicates that it is already old or dirty, or both. Milk known to be gassy, or in which conditions point to gas formation, should be rejected.

The addition of acid or strong starters in quantity sufficient to bring clean, fresh milk at once to the acidity desired has been tried, but without satisfactory results. Success in making Camembert cheese calls for the development of acidity by the development of the typical lactic organisms rapidly enough to prevent unfavorable fermentation. The bacteria added in very sour starters seem to require

^aThe percentages of acidity given in this paper are obtained by titration of 17.6 c. c. of milk to phenolphthalein with n/10 sodium hydroxid. The number of cubic centimeters of alkali required divided by 20 gives the percentage of acidity calculated as lactic acid.

some time to adjust themselves to growth in fresh milk. If in this same time the milk is curdled and partially drained of whey, which carries out with it a large part of the milk sugar, the souring process does not seem to go on normally. We seek by the addition of a starter to give the organisms desired a thorough distribution through the milk and to produce very quickly a preponderance at least of numbers over the undesirable species. In these experiments the advantage seemed to have been thrown away when rennet was added without interposing a short ripening period. Whether the failure in such a case is due to incomplete distribution of the organisms or to the changed composition of the milk due to curdling and draining is not determined.

The presence of the organisms producing gassy curd is very common in Camembert cheese. Many of the market cheeses, both domestic and imported, show more or less gas when cut. Although good cheeses often show traces of gas, the presence of many gas holes in Camembert lowers its quality. A careful study of the prevalence of gas formation and its control seems very necessary to success.

THE PREVALENCE OF GASSY CURD AT CERTAIN SEASONS.

For three successive winters—1907, 1908, and 1909—gassy fermentations during January, February, and March have been found and shown to be due to the prevalence of the coli-aerogenes group of bacteria (identified by Prof. W. M. Esten) in the milk during these months. Incubation experiments with samples of milk during portions of the winter of 1909 failed to develop normal smooth curd in any large percentage of samples studied. Experimental manufacture of Camembert cheese during the months mentioned for the three years was seriously interfered with by the constant appearance of these organisms. The coli-aerogenes bacteria produce gas holes of varying size in Camembert curd and give to the newly made cheese an offensive odor. In these same months of each year the normal activity of the typical lactic species (*Bacillus lactis acidi*), which usually reduces or entirely eliminates the gassy fermentation, failed to develop in untreated milk. Examination of the product of certain factories in New York State showed the same conditions to be prevalent there during the same seasons. Imported cheeses purchased for examination commonly showed more or less of the same trouble. There seems, therefore, to have been a periodical and perhaps a seasonal failure in the activity of the normal souring organism, even when present.

In general, this has resulted in the dominance of the gas-producing type during the first twenty-four hours at least; that is, during the draining period of this kind of cheese. This is long enough to injure

the texture and odor of the newly made cheese. Such gassy cheese often settles together in the succeeding days so that the gas holes are closed and temporarily nearly obliterated. In such cases the typical lactic forms have probably become dominant as the acidity of the cheese increased. After the milk sugar is used up the activity of both forms ceases, or nearly so. In the later stages of ripening, however, after the cheese has begun to soften, the gas organisms seem to resume activity and render the ripe cheese noticeably gassy and often seriously bad-flavored.

During the gassy period the methods which produce good drainage and smooth-textured cheese during the remainder of the year have failed to be effective, either in our experimental work or in factory practice, as judged by the cheeses seen in many cases.

A series of experiments was made to devise means of eliminating or controlling the gassy conditions. Some of these are tabulated as follows:

TABLE 2.—*Results of starter experiments by C. J. Grant.*

Experiment No.	Starter.	Acidity of milk and starter.	Hours ripened.	Final acidity.	Temperature—		Results.
					At beginning.	At end.	
	Per cent.	Per cent.		Per cent.	° F.	° F.	
1830.....	0.0		(a) 0	0.17			Very gassy.
1831.....	.0			.18	Below 60.		Do.
1834.....	4.0	0.225	1½	.255			No gas.
1835.....	10.0	.25	1½	.30			Do.
1836.....	1.8	.19	24	.31	b 66		Do.
1837.....	5.6	.225	24	.44	b 66		Do.
1840.....	.1	.18	24	.20	50		Very little gas.
1841.....	.3	.18	27½	.215	50		Do.
1842.....	.1		24	.25	60	60	Slightly gassy.
1843.....	.3		24	.27	80	60	More than 1842.
1867.....	2.0		24	.225	52		Trace of gas.
1870.....	1.0	.18	24	.245	58	58	No gas.
1872.....	1.0	.185	24	.23	58	58	Trace only of gas.
1875.....	.5		24	.275	60	58	Do.
2358 c.....	1.0	.18	16	.18	44	50	No gas.
2358.....	1.0	.18	16	.21	51	54	Do.
2360.....	1.0	.18	16	.215	43	54	Do.
2362.....	1.0	.18	16	.20	42	53	Do.
2364.....	1.0	.18	16	.20	45	54	Do.

* Stood in refrigerator forty-eight hours.

† Acidity developed very rapidly in five hours. Put in refrigerator the remaining time.

‡ The last five experiments were made to show the relation of temperature to the production of acidity. The gassy season was already past.

Our work indicates that much more care should be given to the production of a starter in these cold months. Repeated preparation of pasteurized milk by methods satisfactory at other seasons showed traces of gas after inoculation with cultures and incubation. When, however, the skim milk used was boiled no difficulty was found in obtaining normal souring. As shown in the table, the starter was added in amounts varying from 0.5 to 10 per cent, and the milk was ripened for various periods after the addition of the starter.

In these experiments it was readily shown that the addition of 3 per cent or more of strong, active starter would reduce the gas formation to a negligible amount if a comparatively short ripening period were used. In many cases, however, the acid added in the starter, together with that developed in ripening, affected the texture of the cheese too greatly. Milk titrating from 0.24 to 0.25 per cent acidity nearly always produces a rough or mealy curd. It is difficult to employ the larger percentages of starter, because they tend to raise the acidity too greatly. Other experiments have shown that a smaller amount of starter (0.5 per cent, or sometimes less) acting for a longer period will produce what may be called a protective ripening; that is, inhibit gas without too great a rise in the titration figure. An experienced factory manager has said that he regards 0.20 per cent acid by titration as a conservative limit to ripening. This figure is, in our experiments, too low to prevent gas formation during the winter months. Examination of cheeses from the same manager's factory for successive seasons showed that his work also suffered from gas during each winter.

As noted above, the same trouble has been seen in varying degrees in a large proportion of the domestic and much of the imported cheese during the winter months. When gassy troubles occur there appears, therefore, good reason for recommending that the acidity at renneting time should be raised to about 0.23 per cent. Long series of experiments indicate that milk can be handled at this acidity without injuring the texture of the resulting curd.

AMOUNT OF STARTER NECESSARY FOR RIPENING MILK.

The length of the ripening period desirable, as well as the amount of starter, must depend upon the conditions. The same result can be reached in different ways. If milk well cooled and free from taint is received during the day and kept over night, ripening may be controlled by a minimum amount of starter (0.5 per cent or even less, perhaps) added at night to milk kept below 57° F. If the temperature goes higher, too high acidity may be expected. The same good result has been obtained by adding about 3 per cent of good starter when the milk is heated for cheesemaking and letting it ripen at 85 degrees until it has developed acidity to from 0.22 to 0.23 per cent, but in this case it must be watched to avoid too high acidity. In using starters it is quite generally agreed that a fresh starter already quite sour to taste but not curdled is preferable to the same starter after curdling. The organisms in the sour but not curdled starter appear to adjust themselves more readily to the fresh milk and to produce acidity more quickly.

DETAILS OF CHEESEMAKING.

Temperature.—The mixed milk is ordinarily heated to 85 or 86° F. The limits of satisfactory work are probably from 84 to 90° F. If color is to be added, this should be done before the milk leaves the mixing vat.

Setting and rennet.—When heated, the milk is distributed into curdling cans. In factories the milk is often piped from the vats through tin or tin-lined pipes running above the row of cans, with a cock opposite each can. Special forms of apparatus are a convenience but not necessary.

The milk is now ready to “set.” For this purpose any standard form of rennet may be used. Rather more rennet is desirable than for American Cheddar cheese. Calculated on a basis of 100 pounds of milk, from 10 to 15 cubic centimeters of commercial liquid rennet (3 to 5 ounces per 1,000 pounds) may be required to obtain the proper texture of curd, according to the conditions of work. This calculation assumes the use of good clean milk not over eighteen hours old, testing when received not more than from 0.16 to 0.18 per cent acid.

Considerable disagreement is found in the recommendations as to the amount of rennet, the temperature to use, the acidity, and the length of curdling time desirable for Camembert. In experimental work different practices have been made to yield cheeses so closely alike as to baffle the description of their differences. In general, comparatively large amounts of rennet have given firmer curds and produced better textured cheeses than when minimum amounts were used. Such cheeses drain more rapidly at first, but retain more water when drainage finally ceases, than those made with the smallest amount of rennet which will permit working. The smaller quantity of rennet produces soft curd which drains more slowly, but ultimately drains lower than the other and makes hard, dry cheeses. The strength of the rennet, the acidity and composition of the milk, and the local conditions are variable factors. The cheesemaker must have in mind the ideal condition of his curd and adjust his own practice to approximate that ideal.

The directions given here do not attempt to settle disputed questions about rennet and its effects. They do, however, represent accepted practices which have also given satisfactory results in experimental work.

Curdling time.—Curd should be ready to “dip” in from one and one-fourth to one and one-half hours; some prefer even a longer time. This will be indicated by the curd beginning to “sweat,” shown by the appearance of drops of water (whey) scattered over the surface of the mass of curd. These drops soon form into a thin

sheet of whey upon the surface. This whey if tested for acidity as it separates usually tests from 0.02 to 0.05 per cent less than the milk at setting time. The curd is now ready to "dip," and should be smooth-textured and quite firm. Cans of curd should not stand long after they are ready to dip. If curd stands a long time in the whey it may become tough or sour, or may cool to a temperature which seriously delays draining, according to conditions. If large amounts of milk are to be handled, the cans should be renneted or set in series so that they become ready to dip as needed.

Cutting the curd.—Some makers cut the curd slightly. In cutting they use a curd knife designed to make circular cuts in the mass. Although such curd drains more rapidly, very little advantage can be claimed for cutting curd at all if working conditions are what they should be.^a In most experiments the advantages in texture, flavor, and handling have favored curd handled without cutting, and this is the practice most generally observed in the best factories.

Arrangement of the hoops.—While milk is curdling the matting is spread upon the draining table, and the hoops are arranged upon the matting as closely as possible. The whole should then be thoroughly wet with warm water so that table and matting shall be wet when dipping begins. If the matting is not wet the curd sticks to it and causes trouble and loss from breaking the cheeses when they are turned the next day.

Dipping.—When a can of curd is ready to dip, the truck is brought into position beside the shelf, and the can gently swung upon it. The truck is then pushed into the aisle between the tables so that the edge of the can comes as close as possible to the tops of the hoops upon the table. In dipping, each dipperful of curd is lowered into the hoop and emptied without loss of time and with as little breaking as possible. One dipperful each is put into a series of hoops, and the process repeated until each hoop contains the required amount of curd. This allows time for partial drainage between dipperfuls.

To obtain cheeses of the desired size with the regular size of hoop it is often necessary to fill up the hoops again after the curd has drained an hour or two. Some experiments indicate that the same result would be more economically reached by making the hoops about half an inch higher. French factories report that 2 liters of milk are sufficient to make a cheese of the usual size. With common factory milk in this country 5 pounds have been reported as necessary to produce the same size of cheese. It is important that the cheeses be uniform—that is, that the total amount of milk be evenly

^a Cutting curd to hasten drainage is a recourse which sometimes aids work with too cold rooms or rooms which become cold at night. When possible the condition should be corrected in such cases as soon as possible instead of changing the practice.

distributed into the proper number of hoops. The time required to do this will vary from day to day because of variations in the condition of the curd, but this should not be permitted to affect the size of the cheeses.

Two workmen to each can of curd will insure that the whole can will be emptied so quickly that breaking and draining in the can are reduced to a minimum. When a can is emptied more slowly, some of the curd becomes broken and hardens rapidly, with a tendency to the production of uneven texture in the cheeses. Quick and careful handling produces the best results.

Draining.—In a room at approximately 68° F. proper draining will require about eighteen hours before the cheeses are solid enough to turn without breaking. In this time they should have drained to less than 2 inches in thickness—perhaps 1½ inches. When ready to turn, the cheese should have a sort of elastic softness, tenacious enough to permit turning with the hand without the removal of the hoop. This turning is usually done the first thing in the morning of the day after the cheese is made. If the room is dry, some cheeses in draining will adhere to the hoops, causing a thick edge and a “dishing” of the center, which sometimes is only half the thickness of the edge. While this is especially liable to occur in a dry room, it may also happen if the hoops are rough or rusty inside or if the holes are too large. A smooth tinned surface with very small holes seems to reduce the trouble greatly without the smallness of the holes checking the drainage.

Trimming.—Rough edges may be trimmed with a knife or an instrument designed for the purpose consisting of a round disk with sharp edges attached at the center to the end of a round handle.

After dipping, in some factories, disks of heavy tinned iron which fit the hoop closely are dropped upon all the freshly dipped cheeses. These disks are said to prevent unevenness of surfaces. They exert a slight but continuous pressure upon the curd. A sucking disk of rubber on the end of a handle is used to remove these in the morning.

Salting.—After turning, the cheeses drain for several hours upon the same mat in the place in which they were made. When solid enough to stand handling, and as the workmen have time, the cheeses are salted. Various methods are in vogue. Some cheesemakers take one or two cheeses in the hands and roll them in salt, edges and both sides at the same salting. Others carefully sprinkle salt on the upper surface and the edges at one time and salt the other surface at a second salting half a day to a day later. Many makers object to handling or rubbing the surface of the cheese while salting. In this practice the cheese is touched as little as possible and only on the edges. Others pay no attention to the details of handling. We have

found little advantage in any specific form of manipulation in salting.

The salt used is usually coarse grained and thoroughly dry. When salt is applied to the surface of a cheese, water (whey) is extracted by the salt. A large part of the salt flows off in this whey and is lost. Some of it, however, diffuses into the cheese. The taste for salt in cheese differs greatly. Different makers of cheese respond to this demand by using different amounts.

The salting establishes a rind upon the fresh cheese. On this rind the molds and bacteria develop afterwards. Many theories are heard as to the relationship of salting to draining and to the growth of the ripening organisms. Proofs of particular views are difficult to obtain because the conditions under which each maker has developed his own view have never been adequately defined. Experiments show that the ripening of the cheese is closely dependent upon its water content and the ripening conditions. The balance between these conditions differs in different factories, but may still be adjusted to obtain good final ripening. No one has been able, therefore, to test all these theories.

After salting, the cheeses are placed upon salting boards, where they remain until they go to the curing room (halloir). The boards are first conveniently rested upon the draining table, with the edges next to the aisle supported by the raised edge of the table, so that the workman can grasp the edges of the board. If space is needed, the salting boards are next raised to the shelf above the draining table, where they remain for the final day of draining. In this way the draining table is cleared for cheesemaking without removing the cheeses from the room. It is possible to use the same tables on successive days for making cheese by removing cheeses to the shelves above in the morning and hurrying the salting process, but larger table space permitting half the table surface to be used for making cheese each day seems an economy of labor.

Draining after salting.—If drainage goes on properly, a cheese should be dry enough to salt eighteen hours after it has been dipped (on the morning after making), or even in less time. A cheese that is wet after eighteen hours (on the morning after making) will probably be ready to salt by the afternoon of that day. The drier cheese salted the second day should stand in the making room until the third afternoon—that is, about thirty hours after salting. If, however, the cheese is wet at eighteen hours old and salting is delayed until it is twenty-four hours old or longer, it should stand after salting at least twelve hours longer than the other—until, say, forty-two hours after salting. No absolute time for draining can be stated, but the figures given may be regarded as desirable intervals found in practice under the conditions named.

Before leaving the making room the cheese should be solid enough so that there is no tendency for the fingers to dent the edges of the cheese when picking it up.

INOCULATION WITH CAMEMBERT MOLD.

If the cheese is to be inoculated at all with the Camembert mold, this should be done just before salting. Inoculation with mold spores, however, has not been practiced in Camembert cheese factories. Once in the factory, the mold has been left to propagate itself. Examination of cultures from most widely separate sources indicates, however, that this mold is not native in America, although there is no difficulty in propagating it here. There seems to be excellent reason for introducing this mold by definite inoculation when new factories are established. Once introduced, so long as proper conditions are maintained, the mold propagates itself so well, as a rule, that inoculation of the fresh cheeses from day to day with pure cultures is probably unnecessary.

When pure cultures are found necessary it would be best to procure them from some reliable laboratory. They can, however, be prepared at home by anyone slightly familiar with the methods of culture used in bacteriology and mycology. When procured they may be used as follows:

Take a small jar with a tin cover which has been punched full of small holes (or an ordinary pepper box). Fill it half full of water, add a piece of moldy cracker or a piece of cheese with a good growth of the proper mold, and shake thoroughly. The contents of the jar are now sprinkled upon the surface of the cheeses, which are then turned and inoculated in the same manner on the other side.

Mold for inoculating.—For those desiring to prepare inoculating material the following practice is recommended: Obtain the hard, dry "water cracker" ("milk crackers" are not satisfactory). Fill quart fruit jars with these crackers and screw on the covers loosely without rubbers. Bake in an oven about two hours (in a laboratory dry sterilize at 140° C. for one hour or more). Care should be used not to burn the crackers. The spores can be transferred directly with a sterilized needle from a stock culture, which should be procured from a reliable laboratory, or they may be put first into sterile water. Each quart jar requires about 3 ounces (100 c. c.) of sterilized water to which 5 to 10 per cent of lactic or tartaric acid has been added (or the water may be boiled thoroughly in a flask plugged with cotton). After cooling, this water may have the mold spores put into it and then be poured into the jar (precautions being used to keep out contamination), when one side only of the cover is raised sufficiently. Roll the jar in the hands to wet all the crackers. When the crackers are all wet, pour off the excess water before they soften

into a pasty mass. Set away at living-room temperature (70° F.). The crackers should be well covered with cottony white mold in ten days. The gray-green color of ripe spores which follows in a few days indicates that the crackers are ready for use.

THE RIPENING OF CAMEMBERT CHEESE.

In factories in France and in those established by French cheese-makers in this country the cheeses are made in a ground-floor room, as a rule, then carried to a second-floor room just above the making room. (See fig. 2.) This first ripening room is furnished with



FIG. 2.—Camembert cheese factory at Lisleux, France. The square windows are seen in the second-floor rooms which are used in the first two weeks of ripening. (From Twenty-second Annual Report, Bureau of Animal Industry.)

windows upon two sides, at least, to provide facilities for rapid ventilation. Various names are given to this room, one of the commonest of which is "halloir." It is characterized by ample provision for ventilation. In our climate, with its extremes of heat and cold, the windows have outer and inner sash, both hinged, making possible free ventilation when wanted and the control of ventilation or of heating and cooling in accordance with changes in the weather. These windows may be large and run from floor to ceiling, or may be small rectangular openings scattered over the whole side of the room. In all cases they must be closely screened to exclude the small flies which are so serious a pest in cheese work.

The humidity in these rooms, as observed, has varied from complete saturation to a condition permitting rather rapid evaporation and shrinkage of the cheeses. The prevention of one or both of these extremes is one of the common difficulties.

A factory manager of experience puts the proper time in the halloir, or first room, at ten to thirteen days. In the further ripening several practices are found. The ideal French practice, according to



FIG. 3.—“Halloir” or first ripening room in American Camembert factory, showing arrangement of shelves and cheeses upon them.

the same manager, transfers the cheese from the first room to the “sechoir” (second or drying room) as soon as the moldy rind with traces of bacterial slime is properly established. In this room the ventilating windows are opened and the evaporation of the extra moisture is accomplished. The cheeses are shrunk from 1 to 3 ounces in weight and reduced in size until they exactly fill the boxes. They are then packed and crated for further ripening. To insure ideal

conditions such cheeses should now go to a ripening cellar to be finished for market. When they come from the second room, softening should have just begun. In the ripening cellar evaporation should be but slight, and the further ripening should be carried as near completion as the market will permit before shipment.

In actual practice, however, makers both in America and in France have often used but one room, the so-called "halloir," in



FIG. 4.—"Sechoir," second, or drying room in American factory, arranged as in a French factory.

which atmospheric conditions have been kept sufficiently dry to bring the cheese to the desired size and appearance in about two weeks in warmer parts of the season. The cheeses are then boxed and crated for ripening or for market. The difficulty of obtaining the desired conditions in the two rooms has often led to this substitution of one for two rooms, with very commonly a resultant loss of character to the cheese. Either the room is too dry, which produces cheese lacking

in moldy covering, shrinking and becoming hard too rapidly, or it is kept too wet, so that ripening develops very rapidly, and the cheeses must be sold partly ripe or lost. Either extreme changes the character of the ripening. In both cases the tendency has been to box and pack the cheeses while still containing too much water, which has led to unpleasant odors and unsatisfactory appearance in the ripened product. Since the market demand for cheeses fully ripe has more and more superseded the trade in half-ripe cheese in America, it has become increasingly difficult to run factories as at present arranged.



FIG. 5.—Another part of the French factory shown in fig. 2. Observe long windows in "sechoir" at right. (From Twenty-second Annual Report, Bureau of Animal Industry.)

EQUIPMENT OF RIPENING ROOMS.

For ripening Camembert cheeses a particular form of shelves has been developed. The permanent part of these consists of posts from floor to ceiling of 2 by 2 or 2 by 4 lumber, in sets of four, 5 to 6 feet apart. In each group the posts are connected in pairs by permanent crossbars of similar size about 1 foot apart, from floor to ceiling, nailed or bolted to the inside of the posts, as shown in figure 3. Frames of strong lumber are made to fit exactly between these uprights resting on the crossbars. These frames are composed of strong side and end pieces and lighter cross strips.

Each frame is covered by a piece of coarse matting ("clayons"). This consists of thin round strips of wood held 1 to 1½ inches apart

by wire strands. The cheeses lie directly upon this matting. A cheese will rest upon three or four strips so that the surface is almost entirely exposed to the air. Such frames carry about 90 cheeses each. Two frames exactly fill the area between four posts, so that all the cheeses are within reach from the sides.

Windows well screened should provide abundant light for working in these rooms. Artificial light (aside from electric) is undesirable because of vitiating the air. There is no advantage in dark rooms, because experiments indicate that the trouble from fly maggots is greater under dark conditions than in fairly well lighted rooms.

Ripening boards.—Smooth boards 8 to 9 inches wide and exactly long enough to rest upon the same supports are used to replace the



FIG. 6.—Another French factory, showing large windows, with blinds, in "sechoir" on second floor. (From Twenty-second Annual Report, Bureau of Animal Industry.)

frames and the coarse or grating-like matting during the later stages. These boards are wide enough to carry two rows of cheeses, and they are smooth to avoid the tendency of the cheese to stick to the wood. The cheeses should be removed to the boards before softening begins. If left upon the mats (clayons) the strips of wood begin to cut into the ripened cheese as soon as softening commences.

Before making any recommendations about factory construction we must first discuss the problems and conditions of ripening, so far as they have been worked out. Factory construction must supply these conditions as closely as possible.

THE NEWLY MADE CHEESE.

Let us first examine the newly made cheese. At twenty-four hours old such a cheese commonly contains from 60 to 70 per cent of water.

It should contain a little less than 60 per cent after salting is completed and the cheese is ready for the ripening process.

As indicated in the discussion of Table 1, a cheese ready for market contains about 50 per cent of water. During the ripening process, therefore, the cheese must lose about 10 per cent of its weight. In actual practice the composition of newly made cheeses will vary considerably from day to day under the most careful management. In most cases these variations are due almost entirely to the rate and amount of drainage. The weight of different cheeses and different brands of cheese in the market runs from 10 to 12 ounces. The amount of loss of weight during the entire ripening process varies, probably, from 8 to 12 per cent; that is, from 1 to $1\frac{1}{2}$ or even 2 ounces for each cheese. Attempts to eliminate this water in the making process have not thus far in our work produced cheeses of the best texture and flavor. The presence of part, at least, of this extra water in the earlier stages of ripening appears to have some necessary relation to the proper development of the ripening agents and to their action.

A ripening process to be successful, therefore, must take into account the composition of the freshly made cheese, the changes of this composition sought in the fully ripe cheese, and the biological conditions under which those changes can be produced. During the ripening process, therefore, the factors to be watched become very largely biological. The details of handling must be based upon an appreciation of the proper appearance and feeling of the cheese at its various stages of ripening. A brief consideration of the agents of ripening and their several parts in the ripening process must be introduced here.

THE RIPENING AGENTS.

The organisms concerned in Camembert cheese ripening have been discussed in previous papers.^a Within the cheese, under normal conditions, the lactic organisms are always the most numerous species present. Other species in smaller numbers are found in freshly made as well as in fully ripe cheeses. Mazé attributes to the lactic organisms not only the souring of the curd but part of the proteolytic action in cheese ripening. This latter effect is said to begin after other agents have reduced the acidity first produced. Of the other organisms present no species so far studied has shown by its numbers, by the uniformity of its presence, or by its effects when introduced into experimental cheeses that it bears any important relation to cheese ripening. The souring of the curd and the production of certain flavors by the continued action of particular races or varieties of lactic bacteria are the changes that have been surely attributed by our work to presence of the bacteria inside the cheese.

^a Bulletins 82 and 109, Bureau of Animal Industry.

The processes which transform the curd in three or four weeks from the hard, sour, undigested condition into the soft, smooth, buttery consistency of ripe cheese appear, therefore, to be attributable to the organisms found in the rind; that is, in the surface one-eighth of an inch or less. The species present are the Camembert mold (*Penicillium camemberti*, or its white form, *P. camemberti* var. *rogeri* Thom), *Oidium lactis*, and the species of bacteria which, with *Oidium lactis*, make up the reddish slime so commonly found upon the surface in the later stages of ripening. Other studies (by Dox and Thom) have shown that the characteristic appearances of ripe Camembert are due to very complete chemical changes of the casein; the fat is little affected. The Camembert mold (*P. camemberti*) has been shown to produce enzymes capable of causing these textural changes in the required time, but not capable of producing the flavors found. Other researches by various authors show that *Oidium lactis* acting alone is able to cause more or less similar chemical changes, but that the texture produced is different. The *oidium*, however, is shown to produce a flavor which forms part, at least, of the characteristic flavor of Camembert cheese. This organism forms a considerable part of the rind in all cheeses studied. It penetrates rather more deeply than the regular Camembert mold. Its presence has been demonstrated in the thin white layer commonly seen just under the rind of old cheeses. Together with the several species of bacteria it is found also in the reddish slime, of which it is always a part.

The part played in cheese ripening by the several species of bacteria found in this slime has never been fully worked out. The presence of the reddish slime covering, or partly covering, the cheese in its later stages of ripening is generally found associated with the presence of excellent flavors and textures. So close is this correlation that the presence and proper development of the red color is a common basis for judging cheese in the factory and in the market. Excellent textures can be found, however, in cheeses entirely lacking the slimy covering, but such cheeses are either mild in flavor or at best lack uniformity. Without proving its exact function in the process, the presence of the slime is certainly associated with or is a result of the conditions under which the best cheeses ripen.

Mazé attributes to the organisms of the rind (molds and bacteria together) the neutralization or destruction of the acidity of the curd. He considers their proteolytic action, although admitted to be present, undesirable in character, and therefore to be kept as small as possible. This view of cheese ripening attributes the production of ammonia to the organisms of the rind, together with minute amounts of substances which give flavor to the curd. The ammonia is said,

then, to neutralize acidity and assist in dissolving the casein, but the bacteria inside the cheese are regarded as the chief agents of the best ripening. Aside from this the organisms of the rind are said to form a necessary protective coating which prevents the access of air to the cheese and so prevents the odors and flavors of rancidity.

Several species are present in all cases, although the exact determination of the part each plays in the ripening of a cheese is not fully known. Cheeses can be ripened to approximately the same appearance while differing markedly in the balance maintained between the various species. It is most probable that the result is more or less composite in every case. Certain conditions are, however, very definitely referable to a predominance of particular species. A particular flavor and texture, such as has been obtained with considerable uniformity by the makers of certain brands, represents a fairly uniform balance of the activities of these organisms due to uniform handling by makers and dealers for long periods of time.

OÏDIUM RIPENING.

Upon Camembert curd *Oidium lactis* will spread over the surface of a cheese within the first forty-eight hours in a warm room. If permitted uninterrupted growth this organism will, inside the first week, produce irregular ridges and wrinkles upon the surface of the mass with a layer of liquefied cheese below this. This liquid layer develops a high flavor. The same conditions which permit this rapid development of *Oidium lactis* favor the development of innumerable yeasts and bacteria upon the rind of the cheese with the oïdium. These bacteria give a yellowish color to the surface and produce strong and often offensive odors.

Such a rind is thin, breaks easily, and peels off, hence must be handled carefully or the cheese is lost. Continued action of the oïdium without Camembert mold may increase the liquid layer, but fails to produce a smooth texture to the center of the cheese. The growth of an excess of oïdium indicates either cheese with too high a water content at the start, or too wet an atmosphere in the room—even a condition in which a change of air by ventilation causes the deposit of water rather than drying. Oïdium is so nearly always present in milk and milk products that special measures to obtain it do not seem to be necessary. It is associated with the ripening and peculiar flavor of Limburg and d'Isigny as well as Camembert cheese. Experiments and observations in cheese cellars indicate that oïdium will displace Camembert mold in very wet cheeses or in saturated atmosphere, but that proper drainage of the cheese followed by a very gradual evaporation restricts the growth of the oïdium and bacteria more than of the Camembert mold. It is thus possible by

control of conditions to obtain any desired balance between the organisms. If this evaporation goes on too rapidly or the curd is too dry at the start, both of these organisms are so handicapped that the native molds present as spores in nearly all milk develop, to the injury of appearance and flavor.

OTHER ORGANISMS OCCURRING ON CAMEMBERT CHEESE.

In addition to the organisms necessary to ripening, a large number of species of molds, yeasts and bacteria are constantly found. The essential species are always found upon good cheese, but almost never without more or less admixture of species unnecessary or even very objectionable. Some of these change the appearance of the cheese; others produce odor or flavor. Some, for example the yeasts, may be present in immense numbers without appearing to exert any marked influence upon the ripening process. The presence of these various species and possible damage from them must be kept in mind in every discussion of the handling of milk and milk products.

Of the molds that may appear a few require special mention. Roquefort mold (*Penicillium roqueforti*) is often found on Camembert cheese. When present it gives a bitter flavor to the cheese, offensive to some tastes, appreciated by others. The true flavors of Roquefort cheese produced by this mold do not appear within the ripening time of Camembert. The most troublesome molds are those which give a strongly ammoniacal odor—*Penicillium brevicaulis* and two related varieties. *P. brevicaulis* is recognizable by the yellowish-brown patches formed upon the old cheeses. The varieties are both white or slightly creamy in color. Under wet conditions these form "cottony" patches almost mistakable for mucors. Under dry conditions the spores are produced as a white dust or powder on the surface of the rind. Once learned, the odor immediately betrays the presence of these forms. They are very generally present upon the cheeses imported from France.

No practice observed eliminates such molds entirely, though they are especially offensive upon cheeses when a heavy moldy rind has been developed. If conditions of ripening are properly maintained, the injuries from these other species are at the same time reduced to the minimum.

Green molds sometimes become very numerous in a factory, especially where several lines of work are going on in the same building. The mold spores are extremely light, float in the air, and lodge in inaccessible places. They may be reached and carried down by filling the air with steam. When the steam has condensed, the thorough spraying and washing down of the walls and floors will relieve the trouble. But the organisms regularly infecting the cheese are not

reached by such means. These are avoided only by strict cleanliness of handling and the vigorous destruction of badly infected material.

CONDITIONS OF RIPENING.

The conditions of ripening must permit the proper development of the organisms sought, yet maintain such a balance between their activities that the cheese when ripe will satisfy the trade. Three factors affect the activity of the molds and bacteria during the ripening process: (1) The initial percentage of water present in the cheese, (2) the temperature of the room, and (3) the relative humidity of the atmosphere.

PERCENTAGE OF WATER.

As already noted, cheeses are usually drained to a little less than 60 per cent water during the making and salting process. No two lots drain to exactly the same percentage of water, but tests of cheeses which resembled as closely as possible those seen made in the factories show the cheeses to contain about 10 per cent more water at the beginning than at the end of the ripening. The cheese maker must be able to judge by the feeling of the cheeses how closely they approach such an average condition. When above or below the average in water content special care would be needed to obtain the best results. Comparison of various makes of cheeses indicates that particular factories or groups of factories maintain fairly close conformity to a certain ideal; other factories or groups set the ideal somewhat higher or lower. The resulting cheeses, therefore, as found in the market, show the differences of their handling by contrasting textures, appearances, and often more or less intense flavors.

TEMPERATURE.

Factory observation and experiments agree in fixing the best limits for work at 52 to 58° F. (12 to 15° C.). Although many factories in France make little provision for artificial heat, these limits would include the larger part of the practice where temperature is controlled. If the rooms are colder, development is delayed without advantage. If the rooms are much above 58° F., the growth of all the organisms present becomes disproportionately increased, the proper balance between their activities is lost, and rapid decay may be expected. The selection of temperature within these limits becomes a matter to be determined by local conditions and the judgment of the maker. The more rapid ripening occurs at the higher temperature. Considerable control of results is therefore possible from small changes in temperature.

RELATIVE HUMIDITY.

The humidity of the air in the ripening rooms is, if possible, even more important than the temperature and the initial water content of the cheeses. The percentage of relative humidity controls the rate of the evaporation of water from the cheese. As the humidity of the air in the room approaches saturation (100 per cent) the rate of evaporation from the cheeses diminishes until a point of equilibrium is reached above which no water is lost, or moisture is even condensed upon the surface. At that point the vapor tension of the cheese exactly equals that of the surrounding air. This point of equilibrium differs for cheeses of different water content. It is considerably higher for cheeses at 60 per cent water content than for cheeses at 50 per cent. In one experiment about 150 grams of cheese testing about 65 per cent water evaporated at the rate of 1 gram per day, whereas a similar amount of cheese testing about 10 per cent less lost weight at the rate of only 0.3 gram per day in the same room at a relative humidity approximating 88 per cent. Although the temperature was low, the sample high in water content showed marked signs of decay in ten days under these conditions. A relative humidity of 88 per cent was manifestly too high to handle cheese as wet as this. The other was found in excellent condition.

Cheeses enter the ripening period with about 8 to 10 per cent excess of water, which must be lost during the process. The humidity of the air surrounding the cheeses must therefore be so handled that this excess of water may be removed. Under factory conditions thousands of cheeses are placed in one room. Ventilation must therefore be provided sufficient to carry away large aggregate amounts of water, but must be controlled so that the rate of removal shall not cause shrinkage and hardening. The working temperature ought not to be affected seriously by this ventilation.

If the relative humidity becomes too high, water gathers in beads and drops upon the walls and ceiling and upon the cheeses and bacterial growth becomes much more rapid than mold growth. Mold may even be suppressed entirely. Under these conditions the cheese develops strong odors and tends to liquefaction and decay. Too wet conditions are usually most quickly detected by the presence of loose floccose colonies of white shimmering mold (*Mucor* species).

If evaporation is too rapid, the danger signal is quickly noted by passing the fingers over the edges of the cheeses. Hard knifelike edges indicate too rapid drying. The cheeses should feel moist (not wet) to the very edge. Under too dry conditions patches of green mold appear quickly. Various species of *Penicillium* will grow upon curd. When the conditions are right Camembert mold will overgrow most of the useless or noxious forms. When the air and the curd

become drier this advantage is lost, so that the appearance of green patches becomes an evidence of such dryness.

Between the wet and dry limits described there is considerable latitude in which the results obtained differ greatly with the details of management. The general principles already discussed apply equally to all practices.

Within these limits of humidity two extremes of practice may be described. In one the humidity of the air is kept approximately uniform, so that the fresh cheeses properly drained evaporate somewhat rapidly, but the rate of evaporation gradually falls to a condition of equilibrium by the time the cheeses have reached the proper moisture content. This period should be two to three weeks, according to the temperature used. During the further ripening little, if any, loss of weight should occur. In such a scheme a single room is used—the halloir.

In the other the humidity is kept as high as the condition of the cheeses will permit, until the rind with its organisms becomes well established and reaches the desired balance between mold, oïdium, and reddish bacteria. This requires, according to its advocates, ten to twelve days if successful. Cheeses must be removed from this room as soon, however, as they have reached the proper appearance. They are then taken to the second room, or "sechoir" (drying room), where the remaining excess of water is dried out quickly, so that they reach nearly the same condition in about the same time as in the practice first described. The change in water content is accomplished differently, however. The latter practice on the whole probably produces stronger flavored cheeses than the former. Between these extremes many variations are practiced. The many differences in texture and flavor in the imported cheeses may be accounted for in such differences of handling.

OTHER CONDITIONS.

A detailed discussion of the observations and appearances during the various stages of normal and abnormal ripening will explain many points in the process.

Under normal conditions a cheese will begin to feel "greasy" in two or three days. Examination of its surface will show some growth of oïdium and often various species of yeast. The normal process of souring, if successful, reduces the growth of bacteria (other than lactic species) to a minimum until a later period. By the end of a week in the ripening room colonies of Camembert mold should be definitely visible; within ten days of ripening such colonies if undisturbed will assume the gray-green color indicative of ripe conidia. The best practice calls for a thin or somewhat incom-

plete covering of mold, well distributed, however, over the surface of the cheese. While upon the mats (clayons) in the first room (halloir) this moldy covering is commonly heaviest upon the underside of the cheese if it remains unturned for a considerable time. If allowed to stand on a board during this time the mold will grow so firmly to the wood that the cheese will be broken in removing it. Frequent turning therefore tends to insure well-distributed mold. If a side or any considerable area lacks mold entirely it becomes covered with bacteria and oïdium, which make a greasy, soft rind and lead rapidly to overripeness, bad flavors, and decomposition if water content is still high. On the other hand, if mold is permitted to develop in a dense mat over the whole cheese it produces a ripening of excellent texture, but as a rule one lacking in flavor. Such a cheese is creamy in texture and flavor, but is often found to have none of the characteristic flavor of Camembert. The appearance of the heavy, moldy rind is objected to by many. The flavors sought seem to be attributable to a combination of the effects of the two molds under conditions favoring the development also of slimy bacteria. Successful ripening must depend upon the balancing of the activities of these organisms. If handled perfectly there is very little if any growth of Camembert mold after the first two weeks. As Dox* has shown, after the mold has begun to produce spores there is a rapid escape of the ripening enzyme from the mold into the cheese. Just at this time—the tenth to fourteenth day, according to conditions—the softening of the curd under the moldy rind begins to be noticeable.

THE CLIMATIC FACTOR.

The preceding pages present the general results to be obtained and the approximate limits within which the work can be done. The working equipment for reaching these results remains to be discussed. Aside from the occasional farm, the factory is the unit of production. In building the ripening rooms, the conditions of cheese ripening must be furnished. Either upon the small or the larger scale, a great variety of equipment can be utilized. Where small numbers of cheeses were made in France the work was done in parts of dwelling houses or in outbuildings adapted for the purpose. Better equipment and more uniform results begin to be obtained when the output reaches 200 to 400 cheeses a day, which is perhaps the limit of production without the construction of expensive buildings.

The economies of equipment and administration begin to be possible when the number of cheeses reaches 1,000 to 2,000 a day. The factories built in America have reproduced types of construction common in France. Although large numbers of good cheeses have

* Bulletin 109, Bureau of Animal Industry, U. S. Department of Agriculture.

been made, the work has been attended with many losses which were found difficult to explain. The discussion of ripening conditions already given, however, points to the probable cause of many of these troubles. The biological factors in cheese ripening demand that temperature be kept within quite narrow limits and that the relative humidity be kept quite high, perhaps 85 per cent. Without disturbing temperature or relative humidity, provision must be made to evaporate about 10 per cent of water from every cheese. The aggregate is large if we figure that the factory must accommodate at least twenty days' make (10,000 cheeses for a small factory) and evaporate at least 1 ounce from each. Air already at 80 to 85 saturation takes up little water, hence the rate of change of air must be rapid. The climatic factor in ripening is thus introduced. In general this may be stated as follows: If the atmospheric temperature be higher than the working temperature, the air for ventilation must be cooled. Cooling raises the relative humidity toward the dew-point (saturation). If, on the contrary, the weather be cold, the air must be warmed; but, in heating, air increases its capacity to absorb water. Since the water is not present in such air when it is introduced into a ripening room, water is rapidly absorbed from the cheeses.

To furnish working conditions, both temperature and relative humidity must average closely enough to the limits of cheese ripening to permit of successful adaptation to the demands of the process.

COMPARISON OF AMERICAN AND FRENCH CLIMATIC CONDITIONS.

In seeking a basis for comparing American with French conditions a table has been made from published weather reports of both countries. In the published reports, mean temperature and mean percentages of relative humidity are given for each month of the year. The French figures have been selected from various published tables to show the mean temperature and mean relative humidity of the whole region as completely as possible without complicating the table. Extremes of temperature are given in some cases to indicate the most rigorous conditions to be expected, in order to compare with the American figures which follow. The latter are taken directly from Stockman's paper,^a except that the "mean" column was averaged from the monthly mean maximum and minimum temperatures. When possible the number of years recorded in compiling the figures is indicated in the table.

^a Stockman, W. B. Temperature and Relative Humidity Data. United States Department of Agriculture, Weather Bureau, Bulletin O (W. B. No. 334). 1905.

TABLE 3.—Mean temperature and relative humidity of points in France and in the United States.

	Caen, altitude 60 feet.		Paris, ^a altitude 256 feet.		Ecorcheboeuf, ^b near sea.		Le Havre.	Rouen, altitude 39 feet.
Years observed.....	15	7	15	7			7	15
Month.	Mean temperature. ^c	Mean relative humidity. ^d	Mean temperature.	Mean relative humidity.	Mean temperature.	Mean relative humidity.	Mean relative humidity. ^d	Mean temperature. ^c
	° F.	Per cent.	° F.	Per cent.	° F.	Per cent.	Per cent.	° F.
January.....	39.9	85.0	37.2	87.4	37	88	86.0	39.9
February.....	43.7	84.7	40.2	86.2	40	87	84.0	41.9
March.....	46.2	81.5	44.6	77.0	43	80	80.7	45.1
April.....	51.1	80.0	50.3	71.3	47	78	77.5	50.7
May.....	55.4	81.0	55.4	71.7	51	77	77.3	55.6
June.....	60.8	80.0	61.8	74.9	57	80	77.5	61.5
July.....	63.9	80.7	66.0	75.9	61	80	78.0	65.7
August.....	63.3	82.4	65.0	76.1	61	82	79.0	65.1
September.....	59.0	83.0	59.0	83.5	57	85	81.4	60.1
October.....	51.3	86.0	50.4	87.1	50	84	83.0	50.8
November.....	45.7	86.0	43.2	87.2	43	85	85.0	44.2
December.....	40.3	86.0	37.0	91.1	38	89	87.0	39.5
Mean.....	51.7	82.9	50.8	80.6				51.6
Highest temperature.....					79-92			
Lowest temperature.....					12-3			

	Alençon, altitude 450 feet.	Albany, N. Y., altitude 97 feet.				Binghamton, N. Y., altitude 875 feet.			
Years observed.....	15	26				8			1
Month.	Mean temperature. ^c	Temperature.			Mean relative humidity.	Temperature.			Mean relative humidity.
	° F.	Mean.	Mean maximum.	Mean minimum.	Per cent.	Mean.	Mean maximum.	Mean minimum.	Per cent.
January.....	37.9	23.0	31	15	80.4	25.0	33	17	81
February.....	40.3	24.0	32	16	78.9	23.0	30	16	80
March.....	44.4	32.0	40	24	76.9	33.0	41	25	81
April.....	49.5	47.0	56	38	69.3	45.5	55	36	76
May.....	54.5	59.5	69	50	71.4	57.0	67	47	78
June.....	61.5	68.5	78	59	71.9	68.0	77	55	77
July.....	65.7	73.0	82	64	71.9	72.0	83	61	74
August.....	64.8	71.0	80	62	75.7	69.5	80	59	83
September.....	60.3	63.5	72	55	77.3	62.5	73	51	89
October.....	50.5	51.5	60	43	79.3	52.5	63	42	92
November.....	43.7	39.0	46	32	80.7	38.0	45	31	91
December.....	38.1	29.0	36	22	81.3	28.5	35	22	76
Mean.....	50.9	48.5	57	40	76.2	45.5	57	38	
Highest temperature.....		100° July.				96° July, Aug.			
Lowest temperature.....		-24° Jan.				-26° Jan.			

^a Relative humidity data for Paris are very completely discussed in *Annales du Bureau Central Météorologique de France*, 1880, B. 106.

^b Ecorcheboeuf is 9 miles from the sea, between Rouen and Dieppe. Full data for this station are given by Moureaux in *Annales du Bureau Central Météorologique de France*, 1890, vol. 1.

^c Report of Voyage of H. M. S. *Challenger*, 1873-1876, Physics and Chemistry, vol. 11, pp. 202-204.

^d From *Annales du Bureau Central Météorologique de France*, 1899-1905.

TABLE 3.—*Mean temperature and relative humidity of points in France and in the United States—Continued.*

	Milwaukee, Wis., altitude 671 feet.				San Francisco, Cal., altitude 153 feet.			
Years observed.....	14				14			
Month.	Temperature.			Mean relative humid- ity.	Temperature.			Mean relative humid- ity.
	Mean.	Mean maxi- mum.	Mean mini- mum.		Mean.	Mean maxi- mum.	Mean mini- mum.	
	° F.	° F.	° F.	Per cent.	° F.	° F.	° F.	Per cent.
January.....	20.0	27	13	78.4	50.0	55	45	79.7
February.....	22.5	30	15	77.6	52.5	58	47	77.9
March.....	30.5	37	24	77.6	54.0	60	48	77.5
April.....	44.0	51	37	73.1	55.0	61	49	77.9
May.....	53.5	62	45	71.2	57.0	63	51	79.0
June.....	63.5	72	55	73.4	59.0	66	52	80.1
July.....	70.0	78	62	70.6	59.0	65	53	84.4
August.....	63.5	76	51	72.6	59.0	65	53	85.8
September.....	62.0	70	54	74.4	61.0	68	54	81.1
October.....	50.5	58	43	75.6	59.5	66	53	78.6
November.....	36.0	43	29	76.9	56.5	62	51	77.3
December.....	26.5	33	20	77.4	51.5	56	47	79.7
Mean.....	45.0	53	38	74.9	56.0	62	50	79.9
Highest temperature.	100° July.				100° June.			
Lowest temperature.	-8 to -25° Nov., Mar.				29° Feb.			

A study of this table is instructive. Caen is near the western edge of the section where the largest amount of Camembert is produced, but probably represents a fair average of the working conditions. It will be noted that in no month does the mean temperature fall below 39° F. The lowest average of minima for any month was about 33° F. The mean relative humidity does not fall below 80 per cent, while in the eight busy months—August to April—it is still higher. Ventilation is practically possible by letting outside air enter at open windows at almost any time in the year without introducing freezing temperature or excessively dry air. A small amount of artificial heat will produce the needed temperature at any time. There are but two months of the year when the temperature is too warm for work within the desirable limits already given.

Compare with these the figures for the American cities listed, excepting San Francisco. The averages for September, October, and November are close enough in some cases to those given for Caen to suggest success. In December, January, February, and March our relative humidity, at best, falls several per cent below that of Caen, while the mean air temperature is 10 to 20° F. lower. Examination of the full reports of variations in temperature, of daily maxima and minima, discloses more striking contrasts than the monthly means. Nature has furnished the French factory a set of working conditions which requires only the opening and closing of the windows with at times a moderate amount of artificial heat. San

Francisco alone of the American cities given is found to have climatic conditions even approximating those of Normandy. In the regions represented by the other American cities the mean temperatures from May to September are mostly prohibitive of work because too warm; from September to December they suggest possible success; from December to March mean temperature and often relative humidity are both low. Not only are the means low, but the variations are greater. Cold northwest storms bring conditions much lower and last often a week or more at a time. Factory managers report that such storms mean the drying out and often the ruin of a large part of the cheeses in stock. Such a storm is said to cause the cheeses to shrivel up as if in a warm, dry room. To obtain working conditions the outside air must be warmed at least 10 and often 20 to 30° F. In this process its percentage of relative humidity falls still lower.

A problem may be given to illustrate conditions in a ripening room at Albany under average conditions for January: Mean temperature, 23° F.; mean relative humidity, 80.4 per cent; 1 cubic foot of air saturated (100 per cent) relative humidity at 23° F. contains 1.488 grains of water; at 80.4 per cent, 1.196 grains of water. The same cubic foot of air heated to 50° F. would hold at saturation (100 per cent) 4.076 grains of water. But as introduced it does contain 1.196 grains. Its percentage of relative humidity is therefore $1.196 \div 4.076 = 29.3$. If the working temperature were lowered to 40° F. the corresponding figure would be about 40 per cent relative humidity. Such air introduced into the ripening room absorbs water rapidly until a point of equilibrium is reached. This water comes from the cheeses. It is thus impossible to introduce outside air into these rooms without rapid drying from the lowered relative humidity of the atmosphere. Both the temperature and the relative humidity of the air introduced must be raised within working limits before the rooms can be ventilated without injury to the cheeses.

Instead of free ventilation, as in Normandy, this air under the present practice must be introduced through the making room, where it absorbs moisture, or ventilation must be reduced until the change of air is nicely balanced by the amount of evaporation desired. No instruments have been found which give a practical and immediate check upon the humidity relations in such rooms. Unless the cheese-maker is extremely careful, unfavorable conditions are detected only after their bad effects have been wrought upon the cheeses.

Binghamton is in the same district as the factories which have made Camembert in New York State. One factory reports success during parts of October and November and at no other season. The report given for Binghamton covers a single year, 1898. A glance at these figures shows that only during October and November do

mean temperature and relative humidity both approach the average for Normandy. At any other season the climatic conditions make success only attainable by artificially producing the proper temperature or humidity, or both. Factories built for French conditions have actually produced fine results a few weeks of each year, and caused numerous difficulties and losses at other seasons.

The table brings out, therefore, the contrast between the climatic conditions of dairy sections of the United States and those of Normandy. The question remains, Can Camembert cheese manufacture be made successful in spite of these conditions? The factory manager already quoted has said: "The Camembert season in America is just about six weeks—October 15 to December 1." This assumes a factory built and run as it would be in northern France, but it also results from seven years' experience with that factory. In that time several hundred thousands of Camembert cheeses were made and sold. Large losses year after year led finally to the closing of this factory, which was built and operated originally by a French family, who continued to operate a factory in France during the same time and who have done so since this was closed.

Several other companies have had more or less similar experience and abandoned the effort or curtailed the product on account of similar troubles. The partial successes obtained in these factories have been best secured in the two or three autumn months which are indicated by the table as reproducing French climate most closely. Similarly, in experimental work at Storrs, Conn., excellent results have been obtained in the same months. Aside from this short period, it has been necessary to determine the conditions needed and to produce them or fail to get good cheeses. When the conditions have been right, good cheeses have been readily obtained. At all other times experimental cheeses were lost.

Both factory operations and experimental work thus show that unfavorable climatic conditions in the United States must be overcome during a considerable part of the year before continued success can be hoped for. To overcome these difficulties, either the location of the factory must be determined by the presence of the climatic conditions desired or the construction of the factory itself must make possible the production of those conditions when necessary. The former would permit Camembert cheese making in very few, if any, places in the Eastern States; there is, however, some reason to hope that this would be successful on the Pacific coast. It is already introduced in the neighborhood of San Francisco. If success is to be attained in the Eastern States the factory must be so built as to enable the cheese maker to furnish the conditions required, irrespective of outside temperature or humidity.

CONSTRUCTION OF ROOMS FOR CHEESE RIPENING.

Since French factory construction has failed in America in the hands of experienced Camembert makers from France, some changes are necessary. Such changes must enable the cheesemaker to minimize the effects of sudden and violent changes of temperature during the winter months, and little or no Camembert can be made under prevailing conditions from May to August. To produce this effect two possibilities are open: (1) Buildings upon the present plans but thoroughly protected from cold winds, insulated against heat and cold, and furnished with efficient systems of controlling ventilation; (2) factories with their ripening rooms partly or entirely below the surface of the ground and furnished with equally good apparatus for ventilation.

Both systems offer advantages. The factories at present built are successful part of the time. If better protected against changes of weather and supplied with efficient means of insuring proper moisture conditions, the same buildings may perhaps be used successfully. Without such alterations they appear to have failed as investments. If correction of their failures is possible their use would involve the least change of methods on the part of the workmen. If the whole ripening process be carried on in rooms partly or entirely below ground, the exposure to storm would be reduced, the production of uniform temperatures would be much easier, and the moisture of the soil would aid in maintaining the desired humidity, but means of producing and controlling ventilation would be equally difficult to manage during a large part of the year. Such rooms, if planned, should, if possible, run into the hillside and have opportunity for ample window space for lighting purposes.

In any plan of construction the apparatus (shelving, frames, mats, etc.) used in the French factories has proved its economy and efficiency.

With either choice of general plan, the space for ripening ought to be divided and arranged to enable the production of a series of conditions within the working limits of cheese ripening already discussed. The proposal of simply a "halloir" and a "sechoir" may be doubled to advantage. Instead of one very moist room and another quite dry, the same space may very desirably be divided into three or four rooms offering a series of conditions. One of these rooms, if the series be in a hillside, ought to be moist enough almost but not completely to stop evaporation. From this condition the other rooms may reduce relative humidity somewhat, and the series should have one room approximating the French "sechoir." The driest room or "sechoir," however, must still have the humidity of French atmosphere, which averages 83 to 85 per cent during the

working season. This is a very different figure from our 70 to 80 per cent humidity, which drops much lower yet when we compensate for the necessary heating of this air before it can be introduced. Such a series should probably range in humidity from 90 to 92 per cent in the wettest to 80 to 83 per cent in the driest room.

If we study the conditions in a room full of cheeses we should find the air within that room to have higher relative humidity than the air outside. If we take an average mean humidity of 83 to 85 per cent as the condition in the Camembert region of France, the average "halloir" would probably test between 87 and 90 per cent when filled with cheeses, and at times higher. In order to transplant cheese ripening to America, therefore, we must get relative humidities approximating such figures.

STAGES OF RIPENING.

First two weeks.—Cheeses enter the first room (halloir) on the third day after making. They usually become sticky, with evidence of *Oidium lactis* and often with the smell of yeast, within three or four days. In five to six days the white threads of Camembert mold begin to be seen. In nine to twelve days the colonies of Camembert mold show traces of colored spores. The colonies of Camembert mold appear as patches on the sides and edges or as a light covering well distributed, but they should not form a heavy felt all over. Areas uncovered or only partly covered with mold should show a marked slimy reddish covering. If the mold forms a heavy felt with the dark color of abundant spores it is called "black" and rejected as not first grade. If the room is too wet, *oidium*, yeast, bacteria, and even white piles of *mucor* tend to displace Camembert mold entirely. As the humidity is lowered the activity of the Camembert mold increases proportionately to that of the slime organisms until a condition is reached in which every trace of slime is covered over by felt-like mycelium. Between the very wet condition and the optimum for mold growth we find the best condition for cheese ripening. A heavy covering of mold extracts water from the surface of the cheese and makes the rind too dry to permit the growth of the slime organisms. At its optimum Camembert mold will overgrow any other species which may happen to be present and at the same time dry the rind so that the bacteria and *oidium* are much restrained, at least. If the moisture conditions are reduced from this optimum, the growth of Camembert mold will be reduced gradually until a percentage is reached at which other molds grow equally well. The appearance of colonies of common green molds indicates, therefore, that the air of the room or the cheeses, or both, are dry enough for the shrinkage of the cheese to become noticeable

also. At the end of two weeks' ripening the rind of the cheese should be well established and the first traces of softening usually appear. If the rooms are kept cold, every stage of ripening may require double the amount of time needed at the limits suggested (52 to 56 or 58° F.). Cheeses low in water content require more time than those containing higher percentages of water.

A series of rooms rather than a single "halloir" offers several advantages. The cheeses made from day to day differ somewhat in condition as they are removed from the making room. If all go to one room kept at a theoretical average humidity, some become too wet and others too dry even when the condition of the cheeses which happen to approximate the average moisture content is satisfactory. With a choice of rooms, cheeses firm and possibly overhard may be kept under more moist conditions. The wet cheeses may be in a room with less humidity. Again, different markets call for varying ideals of ripening. Mild flavors and fairly firm textures can be obtained by ripening where a gradual but continuous evaporation is maintained. Moister conditions, with the consequent development of bacteria and oïdium, are associated with stronger cheese. Such cheeses may be ripened very soft or, after ripening is started, spend a few days in a "sechoir," or drying room, and come out with firmer texture. The treatment of the cheese should depend not only upon its texture and appearance, but also upon the ideals sought. With a series of rooms presenting different but known and controlled conditions one lot of cheeses does not need to be ruined to save others. By moving cheeses from room to room a much larger percentage of good results is obtainable than with less provision for control.

Third week.—During the first two weeks little or no changes in the sour curd are noticeable. A piece of litmus paper pressed against a cut cheese will show an acid condition, although at the end of this time the surface layers for perhaps one-eighth of an inch may test alkaline (blue). During the third week the ripening changes usually progress more rapidly, which will be indicated by a softening of the curd just under the rind. The line between sour curd and ripened cheese is a fairly sharp one, as shown by the softening of the texture. The change from an acid to an alkaline reaction can often be shown by pressing litmus paper against the cut edge of a cheese. The soft, well-ripened part in such cheeses reacts alkaline (blue). Ripe cheese is occasionally acid in reaction to litmus. It is usually, however, neutral or alkaline.

When this softening becomes noticeable at the edges of the cheeses they must be removed from the matting. The mold of the rind tends to grow fast to the wood strips, which cut into the cheese as softening begins. If they are not promptly removed areas of rind will peel off and adhere to the matting when the cheeses are removed.

Ripening on boards.—At this time, if further ripening in the same room is desired, the cheeses are placed upon the smooth boards with which the frames and matting are replaced. Where a second room or “sechoir” is used the cheeses now go into this room. They are turned on the boards, usually every day, to secure uniformity of ripening and to avoid losses from adhering to the wood and breaking. At this time the tendency to evaporation and shrinkage in size is very noticeable; hence the second room must be watched closely.

Red areas.—With the softening period and the constant turning of the cheeses on smooth boards the reddish or yellowish slimy areas increase in size until they often more or less completely cover up the moldy part of the rind. This increase calls for more moist conditions for its full development.

The rate of ripening is closely dependent upon temperature. At the low temperatures—50 to 54° F.—mold growth is still fairly rapid, but the rate of ripening is reduced. It is possible at about 60 degrees to produce cheeses almost completely softened at twenty-one to twenty-four days, when at 50 to 54 degrees a cheese may be less than one-half ripe in the same time. Cheeses high in water content ripen most rapidly; when containing less water the cheeses can be held at higher temperatures with less rapid softening.

Three to four weeks.—Cheeses ripened rapidly decay also more rapidly. It is difficult to hold a cheese ripened in twenty-one to twenty-five days for any length of time. In our experiments rapid ripening has been associated with such rapid decay that a ripening under four weeks has seemed to render cheeses too perishable for successful market handling. Many of our cheeses which became entirely soft in twenty-three to twenty-five days developed within two or three days after becoming fully soft ammoniacal odors and the peculiar flavor which one quickly learns to associate with overripeness. On the other hand, where the process has been prolonged to thirty days or more before complete ripeness the cheese retained acceptable flavor and texture for several days longer.

It is thus possible to reach much the same results in several ways. By making the drier cheeses and ripening at slightly higher temperatures we are able to reach good flavors and textures in the proper time without quick decay. Making a slightly wetter cheese and ripening at the lower temperature accomplishes the same result in the season of year when such cheeses can be handled. If the fully ripe cheese contains more than 51 or 52 per cent of water decay is quick and complete as a rule. When the water content is between 47 and 51 per cent, the fully ripe cheese is firmer and resists decay much longer.

Ripening in boxes.—In factory practice the ripening is rarely carried beyond the third week upon boards. Very often the boards are not used at all. In such cases the cheeses from the boards, or after

they are removed from the matting, are wrapped in parchment paper (sometimes also in tin foil) and put in boxes and the boxes crated. Very few cheeses are as much as half ripened throughout when they are wrapped and boxed.

The stage of ripening at which this is done influences the final product considerably, as a rule. In experimental work it has been found that cheeses wrapped and boxed when two-thirds covered with mold (with the mold still white or faintly tinged with green) usually develop the stronger flavors. If the mold is allowed to go further and to cover the cheese completely and become colored before wrapping, the milder flavors are more common. Sometimes tin foil is used in wrapping cheese. This minimizes evaporation, making practically a sealed package, in which little or no mold growth occurs and even inhibits some forms of bacteria. Hard cheeses low in water content may in this way be made to soften completely. In general, tin foil wrapping prevents evaporation, hastens ripening, produces a more nearly liquid cheese, and leads to strong, almost biting, flavors. Some consumers prefer such cheeses, hence there are brands which regularly supply this demand, but the larger part of the trade does not use cheeses wrapped in tin foil.

Cheeses can be ripened fully, with excellent texture and flavor, upon smooth boards without wrapping at all. But as they soften they tend to break open and lose shape, which makes such ripening impracticable for the factory.

After boxing, the further ripening may be completed at the factory in a special room, as described above, or the cheeses are sent at once to market. In France, where the latter practice was observed, the purchasers who wished to supply fully ripe cheese in exactly the right condition unwrapped them and finished the ripening upon smooth boards in a cellar with air nearly saturated and temperature, as already described, perhaps 56 to 58° F.*

Fully ripe cheese.—The proper texture of a fully ripe cheese is a matter of preference. Good flavored cheeses, cheeses even of the same flavor, can be obtained soft enough to “run” when cut, or they may be of the consistency of moderately soft butter.

WHEN TO MARKET THE CHEESE.

The time of packing and shipping cheeses should depend upon the closeness of connection between factory and consumer. In sending cheeses to the general market in France and also in this country, the practice has been to keep the cheese in the factory until the mold has developed and the cheese has begun to show slime, that is, until

* Soft Cheese Studies in Europe. Twenty-second Annual Report, Bureau of Animal Industry, United States Department of Agriculture, p. 90.

the softening has begun slightly—perhaps one-fourth of an inch under the rind. The cheeses are then packed and sent to market. In particular cases (for special patrons or special markets) they have been allowed to ripen further. But in general the maker has endeavored to get the preliminary stages of ripening properly started, and then the cheeses are sent to the distributing center. Once on the market such cheeses either go to special cellars for finishing, which may be in the hands of the commission man or the actual user, as in cases of hotels, cafés, etc., or they may go direct to the retailer and be sold for consumption.

Much observation of the cheeses offered for sale at retail shows that in France very many such cheeses reach the private consumer less than one-third ripe.

THE AMERICAN MARKET.

In America most users of Camembert demand fully ripe cheese. Some prefer it just before complete ripeness, when there is a slight layer of sour curd in the center, while a very few ask for cheese with little or no softening. This complicates the problem of handling Camembert. The average consumer or even the dealer has not understood how to handle it at the various stages of ripeness.

The necessity remains, therefore, for the maker who sends cheese to the general market to send it so long before complete ripeness as to minimize losses from overripeness. At the same time, to sell fully ripe cheese to the consumer, the maker must ripen as far as he dares before selling. Careful study of the condition of the market emphasizes the desirability of the closest possible connection between factory and consumer.

SHALL THE FACTORY MAKE CAMEMBERT ONLY?

In establishing cheese factories it is generally good policy to combine the manufacture of several kinds of dairy products. The standard of milk for Camembert needs to be somewhat higher than is absolutely necessary for some other work. The ability to utilize all milk in reasonably good condition would save loss caused by refusing a patron's milk which might occasionally be unsuitable for Camembert, but could be used for butter or for other cheeses. Further, the factory should be able to take the milk throughout the year, while Camembert has not been handled to advantage in the United States during our hot months of summer. Some combination with other uses of milk should make work practicable throughout the year.

THE COOKING OF CAMEMBERT CHEESE.

That Camembert is a very perishable product has been repeatedly emphasized in these discussions. When fully ripe the marketable period is very short—a few days or a week at best—and even that time requires low temperature and care. It thus happens that losses in the American market have been large. Many of these losses have fallen directly upon the consumer who has bought the cheese at his own risk in the market and found it too ripe for his taste or having some of the flavors of overripeness or too rapid ripening, which are objected to by many persons. Experiments show that any cheese which is in condition to be selected by one even casually acquainted with Camembert can be used acceptably in cooking. The cheese need not, therefore, be entirely lost, even though higher in price than the kinds of cheese usually used in cooking.

Several brands of canned or tinned Camembert are obtainable. All are cooked forms of this cheese and suggest the possibilities of preventing loss of stock in this way. In America all soft-cheese trade has hitherto been irregular and uncertain. Dealers and makers have suffered from irregularity, especially in the demand for Camembert. Cheeses not sold are lost, as a rule. Cooking or canning under proper conditions offers a method of minimizing such losses.

MAKING CAMEMBERT CHEESE ON THE FARM.

As a general market proposition it is not advisable to undertake the making of Camembert cheese on the farm. In special cases, such cheesemaking may undoubtedly be developed to considerable advantage, but as yet these possibilities have not been really touched in this country. The making and ripening of the cheese in a certain household known to the writer has, however, been successfully carried on during the past year, although the cheeses produced have varied considerably in texture and flavor, and all have differed from the imported cheese in appearance. It may be doubted whether the uniformity demanded by the trade at present could be readily obtained when work is done with such small numbers of cheeses. The equipment used costs but a few cents in addition to the utensils already in the home. For ripening, a small zinc-lined refrigerator has proved capable of adaptation to produce approximately the conditions of ripening. With this or similar equipment it has been demonstrated that a busy housekeeper in the intervals of her work can make and ripen Camembert cheese enough to supply her own family and some of her friends.

In this instance the complex problems of producing Camembert cheese were fairly well mastered in a single season. At the same time no losses were necessary in the process, because every cheese was

eaten in some stage or condition of ripeness, while repeated trials showed that Camembert cheeses, either lacking in flavor or too strong, could still be utilized in cooking. Thus the value of the materials used was recovered as acceptable food in the family in every case, since deficiencies in flavor did not prevent the use of the cheese as food. Although this work in its beginnings has cost a disproportionate amount of time for the food actually produced, the housekeeper who has already done this expects to continue to develop this work and to sell the product to advantage in a special market.

Camembert cheesemaking has been discussed in this paper mainly as a factory proposition. The production of this cheese for the general market will probably remain so in this country, just as it has become so in France. It is entirely possible, however, to produce cheeses of this type for home use wherever some member of the family will take the trouble to learn the work properly. The skill and expense entailed are no greater than the demands of many other lines of work already regularly carried on in the household for no larger return. In many special cases production can go further and very profitably supply the special personal market in the same way as many families now regularly supply butter or other products directly to customers.

The cheese made under these conditions would probably be refused if offered in the general market. The general market requires uniformity among large numbers of cheeses. This would not be reached by putting together the cheeses from a large number of farms so well as if all came from one factory. Where a small number of cheeses is made, control of conditions is more difficult. Cheeses will vary more from day to day, and they would require more care in selling than the dealer can afford to give. Products of this kind must be made for and delivered directly to a special market to obtain satisfaction for either party.

In the making of Camembert cheese on a small scale the problems to be met will seem new and strange at first. It will take some time to acquire the skill and judgment to work successfully, and especially to develop a considerable number of workers with such skill. But this is in no way impossible, and there are many situations in which those engaged in dairy work might regularly produce this kind of cheese from surplus milk and add materially to their profits without appreciably increasing their expenses. Like any other line of work worth doing, it must be learned well or it will lose for the investor both the time and money put in. Some markets will take hard cheese either good or bad and pay something for it, but soft cheeses, especially Camembert, are either good or good for nothing in the ordinary market. A family would be able to consume its experimentally made cheese, but not the output upon a larger scale. The conditions

of each case should be well considered before Camembert cheesemaking is undertaken upon the farm. Under proper conditions it may be a source of both pleasure and profit.

In the vast majority of cases better results will be reached for the farm by perfecting the control of the production of milk than by attempting to market the milk produced as Camembert cheese. Comparatively few farms can combine these lines of work to advantage.

ACKNOWLEDGMENTS.

The author desires to acknowledge the assistance of Messrs. A. W. Dox, F. R. Thomson, and C. J. Grant, members of the staff at the Storrs Agricultural Experiment Station, with whom the work described in this bulletin has been discussed in detail. Acknowledgment is also made of conferences with and suggestions from Messrs. A. W. Ferguson and G. H. Garstin and of analyses by Mr. H. D. Edmond. Numerous courtesies have been freely extended by manufacturers of the Camembert and related kinds of cheese in America and by dealers in and importers of French cheese.

PUBLICATIONS RELATING TO CAMEMBERT CHEESE.

The following publications have been issued by the United States Department of Agriculture or by Storrs Agricultural Experiment Station, Storrs, Conn. Those for which a price is shown may be secured only on application and remittance to the Superintendent of Documents, Government Printing Office, Washington, D. C. Others may be had by application to the United States Department of Agriculture, Washington, D. C., or to the Storrs Agricultural Experiment Station, Storrs, Conn., as the case may be.

CONN, HERBERT W.; THOM, CHARLES; BOSWORTH, A. W.; STOCKING, W. A., Jr.; and ISSAJEFF, T. W.

The Camembert type of soft cheese in the United States. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 71. Washington, 1905. Price, 5 cents. Also published as Bulletin 35 of Storrs Agricultural Experiment Station. Storrs, 1905.

DOANE, C. F., and LAWSON, H. W.

Varieties of cheese: Descriptions and analyses. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 105. Washington, 1908. Price, 10 cents.

DOX, ARTHUR W.

Proteolytic changes in the ripening of Camembert cheese. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 109. Washington, 1908. Price, 5 cents.

ISSAJEFF, THEODORE W.

Investigations in the manufacture and curing of cheese. Directions for making the Camembert type of cheese. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 98. Washington, 1907. Price, 5 cents. Also published as Bulletin 46 of Storrs Agricultural Experiment Station. Storrs, 1907.

THOM, CHARLES.

Fungi in cheese ripening: Camembert and Roquefort. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 82. Washington, 1906. Price, 5 cents. Also published in Seventeenth Annual Report of Storrs Agricultural Experiment Station for the year ending June 30, 1905, pp. 73-115. Middletown, 1906.

Soft-cheese studies in Europe. U. S. Department of Agriculture, Bureau of Animal Industry, Twenty-second Annual Report, 1905, pp. 79-109. Washington, 1907.

The care and testing of Camembert cheese. U. S. Department of Agriculture, Bureau of Animal Industry, Twenty-fourth Annual Report. 1907, pp. 339-343. Washington, 1909. Price, 85 cents. Issued separately as Circular 145 of the Bureau of Animal Industry. Also published in New York Produce Review and American Creamery, vol. 25, No. 24, pp. 970-971. New York, April 8, 1908.

Popular descriptions and brief discussions of Camembert cheese are numerous in dairy text-books and journals. Only a few of the more important or more recent references consulted in preparing this paper are listed here. The chemical literature is reviewed and listed by Arthur W. Dox in Bulletin 109, Bureau of Animal Industry, cited above.

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VAN SLYKE, LUCIUS L., and PUBLLOW, CHARLES A.

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Issued August 18, 1909.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY.—BULLETIN 116.
A. D. MELVIN, CHIEF OF BUREAU.

TESTS CONCERNING TUBERCLE BACILLI
IN THE CIRCULATING BLOOD.

BY

E. C. SCHROEDER, M. D. V.,
Superintendent of Experiment Station,
AND

W. E. COTTON,
Expert Assistant at Experiment Station.



WASHINGTON:
GOVERNMENT PRINTING OFFICE.
1909.

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LETTER OF TRANSMITTAL.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY,
Washington, D. C., July 7, 1909.

SIR: I have the honor to transmit herewith, and to recommend for publication in the bulletin series of this Bureau, the accompanying manuscript, entitled "Tests Concerning Tubercle Bacilli in the Circulating Blood," by Dr. E. C. Schroeder and Mr. W. E. Cotton, of the Experiment Station of this Bureau.

The tests described in the bulletin were undertaken upon the appearance of a recent paper in which were recorded a large number of microscopic examinations of the blood of tuberculous individuals, the result of which, it was stated, proved that tuberculosis in all its forms was a bacteriemia.

This conclusion was entirely contrary to the views of the authors of this bulletin, based upon their work in the Bureau for a long series of years; and as the matter was of great importance in its bearing on the tuberculosis question, it was considered advisable to put it to a thorough test. Accordingly inoculation experiments were made with the blood of a large number of tuberculous cattle, and the results have demonstrated that tuberculosis is not in any sense a bacteriemia, and that if tubercle bacilli ever float in the blood of tuberculous animals this is an exceedingly rare condition.

Respectfully,

A. D. MELVIN,
Chief of Bureau.

Hon. JAMES WILSON,
Secretary of Agriculture.

CONTENTS.

	Page
Introduction	7
Summary of present tests	9
Records of cattle and results of guinea-pig inoculations.....	10
Discussion of results	18
Supplemental tests regarding possible immunity.....	20
Conclusions.....	23

TESTS CONCERNING TUBERCLE BACILLI IN THE CIRCULATING BLOOD.

INTRODUCTION.

In a paper dealing with the occurrence of tubercle bacilli in the circulating blood, read before a medical society some months since and soon afterwards published in a medical journal,^a there were recorded the results of microscopic examinations of the blood of 125 tuberculous individuals, some of whom were affected with only incipient tuberculosis, and the statement was made that tubercle bacilli were found in the blood of every one of them. In some cases only a few bacilli were seen, but "they were mostly in large numbers, and clumps of 30 to 40 bacilli were not unusual, especially in cases of acute miliary tuberculosis." From his observations the author of that paper formulated the conclusion: "It appears that tuberculosis in all its forms is a bacteriemia."

That tubercle bacilli occasionally float in the blood stream is hardly open to question, because of the occurrence of isolated lesions in the bodies of otherwise tuberculous as well as otherwise healthy individuals located in regions remote from the various channels that communicate with the exterior. The same is true when we consider cases of more or less generalized tuberculosis with many lesions in widely separated portions of the body and cases of miliary tuberculosis with innumerable lesions of approximately, if not precisely, the same age and stage of development. But such occasional presence of tubercle bacilli in the circulating blood is a very different condition from their constant occurrence in it in sufficient numbers to justify the classification of tuberculosis as a bacteriemia. Hence, Rosenberger's conclusion was received with considerable surprise and doubt.

Although the conclusion seemed sufficiently incredible because of the simple fact that a constant occurrence of tubercle bacilli in the blood of all tuberculous individuals could hardly have been overlooked by the host of investigators who have studied tuberculosis

^a Rosenberger, Randle C., "The presence of tubercle bacilli in the circulating blood in tuberculosis." (Read before the Pathological Society of Philadelphia, December 10, 1908.) *American Journal of the Medical Sciences*, vol. 137, No. 2, pp. 267-269. Philadelphia and New York, February, 1909.

with no greatly different technique than Rosenberger used, we did not feel warranted in opposing it without offering some specific evidence. Because of the important bearing of the matter on the tuberculosis problem, the experiments hereinafter reported were carried out.

Rosenberger stated that he found tubercle bacilli on microscopic examination in the blood of every one of the 125 cases of tuberculosis he studied, notwithstanding that some of the cases were incipient and failed to show tubercle bacilli in the sputum. It was, therefore, almost taken for granted that the microscopic examination of blood, according to his method, of animals affected with advanced and long standing tuberculosis and animals that were expelling tubercle bacilli from their bodies in large numbers would reveal at least a few tubercle bacilli. A considerable number of such microscopic examinations were made, but not a tubercle bacillus was found in our blood preparations, and hence we have to record wholly negative results with the blood of tuberculous animals. Similar negative results were obtained with the blood of tuberculous persons in two large New York hospitals.^a

It is not uncommon for virulent tubercle bacilli to be present in animal substances in numbers too small to serve for their detection by optical methods. For example, at the Experiment Station we found the intraabdominal injection of guinea pigs with suspected milk to be a test for tubercle bacilli that has fully fifty times the delicacy of a microscopic examination. Furthermore, tinctoral and optical methods of distinguishing between tubercle bacilli and other acid-fast bacteria are not wholly satisfactory, hence we concluded to inject a sufficient number of guinea pigs with blood from a sufficient number of certainly tuberculous cattle to show conclusively that tubercle bacilli either are or are not commonly present in such blood.

Incidentally it appears that Doctor Rosenberger failed to confirm adequately by animal experiments his surprising microscopic observations, which, if correct, would have been of the greatest value alone for the early and certain diagnosis of tuberculosis. In all he inoculated only two guinea pigs, of which he gives a record; one with blood from a tuberculous person who was expelling tubercle bacilli per rectum, and one with blood from a case of acute miliary tuberculosis. The development of tuberculosis in the latter guinea pig can not be regarded as a remarkable phenomenon. There is nothing about the fact that a guinea pig contracted tuberculosis after an injection of blood obtained from a case of acute miliary tuberculosis that necessitates a modification of our currently accepted views on the presence of tubercle bacilli in the circulating blood. That is to say, we need not look upon tuberculosis as a bacteriemia because tubercle

^a Editorial in the Medical Record, New York, April 3, 1909, p. 508.

bacilli were demonstrated in blood of a kind in which we have long considered that they might sometimes occur.

This leaves one guinea pig that may have some evidential value, but we must not lose sight of the fact that it was injected with blood obtained from a person who was expelling tubercle bacilli from his body and hence to some extent infecting his environment. We must also bear in mind that guinea pigs are highly susceptible to tubercle bacilli injected into their bodies, and that it is often impossible for an investigator who handles much tuberculous material, who is in frequent contact with tuberculous persons, and whose environment may be characterized as containing tubercle bacilli, to eliminate all danger of extraneous tuberculous infection sufficiently to make a test satisfactory when he seeks to verify the tuberculous character of some material from a tuberculous individual by the injection of one, and only one, guinea pig.

SUMMARY OF PRESENT TESTS.

Our own tests were made entirely with the blood of tuberculous cattle. In every case the blood was drawn from the jugular vein of the tuberculous animal and injected in its fresh, naturally warm state into the peritoneal cavity of a guinea pig. The tuberculous cattle, as their records show, may be divided into four distinct lots, according to their tuberculous condition:

Lot 1. Four cattle, the precise tuberculous condition of which is known, because they were killed and examined post-mortem shortly after blood was drawn from them for guinea-pig injections.

Lot 2. Six cattle, known to be tuberculous because they had reacted with tuberculin, because tubercle bacilli were found in their feces on microscopic examination, and because their feces were proven to be infectious by animal experiments.

Lot 3. Nineteen cattle, known to be tuberculous because they had reacted with tuberculin and because tubercle bacilli were found in their feces on microscopic examination.

Lot 4. Thirteen cattle, known to be tuberculous because they had reacted with tuberculin.

We made no attempt to treat the blood used for the injections in any way, because we assumed that the best results would be obtained with it by transferring it as rapidly as possible from the tuberculous cattle to the peritoneal cavities of the guinea pigs. It was learned from the injections that guinea pigs tolerate a relatively large quantity of bovine blood in their peritoneal cavity. The guinea pigs that died shortly after as the result of the blood injections (about 15 per cent of all injected) with few exceptions showed extreme impaction and some inflammation of the large bowel, associated in several instances with invagination of the colon.

The possibility exists that the intraperitoneal injection of from 3 to 5 cubic centimeters of fresh, warm blood from tuberculous cattle induces an immunity in guinea pigs to the tubercle bacilli the blood may contain. Though this view is purely hypothetical and we know of nothing to sustain it, we have carried out an investigation to prove or disprove it, and will give the results later in this paper.

The total number of cattle from which blood injections were made was 42, and these, as their records show, represent a considerable variety relative to the severity and extent of the tuberculous disease with which they were affected. They ranged from animals that would not have been suspected to be diseased without a tuberculin test to a cow so badly affected that a calf of which she became the mother a little less than a year before her blood was used for guinea-pig injections was born affected with tuberculosis contracted from ante-partum exposure to her tuberculous body.

The total number of guinea pigs injected was 104. Of these, 16 died within a few days after the injection and no doubt as a result of it. Three died of intercurrent affections, but not until a sufficient period of time had passed for lesions of tuberculosis to become clearly manifest. The remaining 85 lived until they were killed after a lapse of from seven and one-half to eleven weeks, or an average for all of seventy days after they were injected. The three guinea pigs that died of intercurrent affections showed no lesions of tuberculosis on post-mortem examination, and 84 of the 85 guinea pigs that lived until they were killed showed no lesions of any kind on autopsy. One guinea pig of the 85 showed lesions very slightly resembling tuberculosis, but these were proved by microscopic examinations and guinea-pig inoculation tests to be free from tubercle bacilli.

A detailed record of the cattle and guinea pigs used in our tests follows:

RECORDS OF CATTLE AND RESULTS OF GUINEA-PIG INOCULATIONS.

The 42 cattle included in the records below are all that were available for this investigation among the tuberculous cattle kept for various purposes at the Bureau of Animal Industry Experiment Station. The general condition of the cattle is briefly defined as good, fairly good, fair, or poor; and as these terms are used somewhat arbitrarily, it is desirable to specify more precisely what they are intended to convey. The word "good" is used in connection with cattle that were really to all appearances in excellent physical condition, and of which no one would suspect that they were diseased. The words "fairly good" are used to mean that condition commonly found among dairy cows of the better class. "Fair" is used to desig-

nate a condition which the average dairyman regards as satisfactory, and "poor" is applied to cattle that are thin or that show visible symptoms of disease.

LOT 1.

Bull 393, general condition very good, had been affected with tuberculosis a year or more; was killed and examined post-mortem April 8, 1909. The autopsy revealed only one small tuberculous lesion located in one of the superficial inguinal glands.

On February 5, 1909, two guinea pigs, Nos. 2891 and 2892, received each an intraabdominal injection of 2 c. c. of blood from the bull. One guinea pig, No. 2891, died on February 14, 1909, affected with invagination of the bowel. The other guinea pig remained healthy until April 13, 1909 (sixty-seven days after injection), when it was killed and found on post-mortem examination to be free from lesions of disease.

Cow 533, general condition poor, had been affected with tuberculosis two years or more; was killed and examined post-mortem April 24, 1909. The autopsy revealed the following conditions: The principal lobe of the right lung contained a cavity about 3 inches in diameter, partly filled with pasty, necrotic tuberculous material. This cavity was in direct communication with a large bronchial tube, which contained a considerable amount of material discharged from the cavity. Sprinkled throughout the lungs generally were a number of smaller tuberculous foci, in a completely broken-down condition. The mediastinal and mesenteric lymph glands and the liver were sprinkled with tuberculous foci, some of which were as much as half an inch in diameter. Prior to the cow's death her feces were examined microscopically on nine different days and on six of these days were found to contain tubercle bacilli.

On February 3, 1909, two guinea pigs, Nos. 2859 and 2860, received each an intraabdominal injection of 3 c. c. of blood of the cow. The guinea pigs remained healthy until April 13, 1909 (sixty-nine days after injection), when they were killed and found on post-mortem examination to be free from lesions of disease.

Cow 549, general condition poor, had been affected with tuberculosis several years. On March 27, 1908, she gave birth to a calf affected with congenital tuberculosis. The cow was killed April 8, 1909, and on autopsy was found to be affected with advanced, generalized tuberculosis. The lungs contained lesions varying from quite recent tuberculous disease to large tuberculous cavities that had discharged most of their contents through the bronchial tubes. No tests were made relative to the infectious character of the feces before death.

On February 3, 1909, two guinea pigs, Nos. 2863 and 2864, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 13, 1909 (sixty-nine days after injection), and on post-mortem examination were found to be free from lesions of disease.

Cow 552, general condition poor, had been affected with tuberculosis several years; was killed April 1, 1909. The autopsy revealed a fairly generalized tuberculosis with lesions of greater or less magnitude in the lungs and in the pharyngeal, bronchial, and mesenteric lymph glands. Prior to the cow's death her feces were examined microscopically on ten different days and on six of these days were found to contain tubercle bacilli.

Hogs that were fed feces from the cow contracted tuberculosis, and guinea pigs inoculated subcutaneously with small masses of her feces likewise contracted tuberculosis.

12 TESTS CONCERNING TUBERCLE BACILLI IN THE BLOOD.

Guinea pigs were injected intraabdominally with blood from this cow as follows:

January 25, 1909, guinea pig 2785 received 5 c. c.
January 25, 1909, guinea pig 2786 received 5 c. c.
January 25, 1909, guinea pig 2783 received 2½ c. c.
January 25, 1909, guinea pig 2784 received 2½ c. c.
January 25, 1909, guinea pig 2781 received 1 c. c.
January 25, 1909, guinea pig 2782 received 1 c. c.
February 3, 1909, guinea pig 2861 received 3 c. c.
February 3, 1909, guinea pig 2862 received 3 c. c.
February 5, 1909, guinea pig 2889 received 2½ c. c.
February 5, 1909, guinea pig 2890 received 2½ c. c.

Guinea pigs 2786 and 2890 died prematurely as a result of the blood injections, and the remaining 8 were killed on the following dates and on autopsy were found to be free from lesions of disease: Guinea pigs 2781 and 2782, killed March 27, 1909 (sixty-one days after injection); guinea pigs 2783 and 2784, killed April 13, 1909 (seventy-eight days after injection); guinea pig 2785, killed April 12, 1909 (seventy-eight days after injection); guinea pig 2889, killed April 13, 1909 (sixty-seven days after injection); guinea pigs 2861 and 2862, killed April 13, 1909 (sixty-nine days after injection).

LOT 2.

Cow 511, general condition poor, had been affected with tuberculosis eighteen months or more. Microscopic examinations of the feces on seven different days revealed tubercle bacilli on three days. A hog fed with feces from the cow contracted tuberculosis.

On February 1, 1909, two guinea pigs, Nos. 2829 and 2830, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs remained healthy until April 13, 1909 (seventy-one days after injection), when they were killed and found on autopsy to be free from lesions of disease.

Cow 537, general condition fairly good, had been affected with tuberculosis more than two years. Microscopic examination of the feces on fifteen days revealed tubercle bacilli on eleven days. Guinea pigs inoculated with small masses of feces contracted tuberculosis.

Guinea pigs were injected intraabdominally with blood from the cow as follows:

February 4, 1909, guinea pig 2871 received 3 c. c.
February 4, 1909, guinea pig 2872 received 3 c. c.
February 19, 1909, guinea pig 3062 received 3 c. c.
February 19, 1909, guinea pig 3063 received 3 c. c.

Guinea pig 2872 died prematurely as a result of the injection. Guinea pig 2871 was killed April 13, 1909 (sixty-eight days after injection), and on autopsy was found to be free from lesions of disease. Guinea pigs 3062 and 3063 were killed April 13, 1909 (fifty-three days after injection), and on autopsy were found to be free from lesions of disease.

Cow 538, general condition very poor, had been affected with tuberculosis two years or longer. Microscopic examinations of feces on eleven different days revealed tubercle bacilli on nine days. Guinea pigs inoculated with small masses of feces and hogs fed feces of this cow contracted tuberculosis.

On February 3, 1909, two guinea pigs, Nos. 2851 and 2852, received each an intraabdominal injection of 3 c. c. of blood from the cow. On April 13, 1909 (sixty-nine days after injection), the guinea pigs were killed and on autopsy were found to be free from lesions of disease.

Cow 555, general condition fairly good, had been affected with tuberculosis more than two years. Microscopic examinations of feces on five different days revealed tubercle bacilli on two days. A hog fed feces from the cow contracted tuberculosis.

On January 30, 1909, two guinea pigs, Nos. 2811 and 2812, received each an intraabdominal injection of 3 c. c. of blood from the cow. Guinea pig 2811 died prematurely as a result of the injection. Guinea pig 2812 was killed April 12, 1909 (seventy-two days after injection), and on post-mortem examination was found to be free from lesions of disease.

Cow 567, general condition good, had been affected with tuberculosis at least two and one-half years. Microscopic examinations of feces on ten different days revealed tubercle bacilli on five days. A hog fed with feces from the cow contracted tuberculosis.

On February 4, 1909, two guinea pigs, Nos. 2869 and 2870, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 12, 1909 (sixty-seven days after injection), and on autopsy were found to be free from lesions of disease.

Cow 646, general condition fair; had been affected with tuberculosis for some time, but just how long was not known. Microscopic examinations of feces on two different days revealed tubercle bacilli on one day. Guinea pigs inoculated with her feces contracted tuberculosis.

On February 2, 1909, two guinea pigs, Nos. 2847 and 2848, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 13, 1909 (seventy days after injection), and on autopsy were found to be free from lesions of disease.

LOT 3.

Cow 503, general condition good; had been affected with tuberculosis at least two and one-half years. Microscopic examinations of feces on two days revealed tubercle bacilli on one day.

On January 30, 1909, two guinea pigs, Nos. 2805 and 2806, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 30, 1909 (seventy-three days after injection), and on autopsy were found to be free from lesions of disease.

Cow 510, general condition fairly good; had been affected with tuberculosis about three years. Microscopic examinations of feces on three days revealed tubercle bacilli on two days.

Guinea pigs were injected intraabdominally with blood from the cow as follows:

January 20, 1909, guinea pig 2791, received 5 c. c.

January 20, 1909, guinea pig 2792, received 5 c. c.

February 4, 1909, guinea pig 2881, received 3 c. c.

February 4, 1909, guinea pig 2882, received 3 c. c.

Guinea pigs 2791 and 2792 died prematurely as a result of the injections. Guinea pigs 2881 and 2882 were killed April 13, 1909 (sixty-eight days after injection), and on autopsy were found to be free from lesions of disease.

Cow 512, general condition good; had been affected with tuberculosis eighteen months or longer. Microscopic examinations of feces on six different days revealed tubercle bacilli on four days.

On February 11, 1909, two guinea pigs, Nos. 2823 and 2824, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 12, 1909 (seventy days after injection), and on autopsy were found to be free from lesions of disease.

Cow 513, general condition fairly good, but had greatly enlarged throat glands; had been affected with tuberculosis eighteen months or longer. Microscopic examinations of feces on four different days revealed tubercle bacilli on two days.

On February 2, 1909, two guinea pigs, Nos. 2835 and 2836, received each an intraabdominal injection of 3 c. c. of blood from the cow. Guinea pig 2835 died prematurely as a result of the injection. Guinea pig 2836 was killed April 12, 1909 (sixty-nine days after injection), and on autopsy was found to be free from lesions of disease.

Cow 514, general condition poor; had been affected with tuberculosis about three years. Microscopic examinations of feces on three different days revealed tubercle bacilli on one day.

On January 30, 1909, two guinea pigs, Nos. 2815 and 2816, received each an intraabdominal injection of 3 c. c. of blood from the cow. Guinea pig 2816 died of an intercurrent affection March 3, 1909 (thirty-two days after injection), and on autopsy was found to be free from lesions of tuberculosis. Guinea pig 2815 was killed April 12, 1909 (seventy-two days after injection), and on autopsy was found to be free from lesions of disease.

Cow 515, general condition fair, had been affected with tuberculosis eighteen months or longer. Microscopic examinations of feces on six different days revealed tubercle bacilli on four days.

On February 1, 1909, two guinea pigs, Nos. 2821 and 2822, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 12, 1909 (seventy days after injection), and on autopsy were found to be free from lesions of disease.

Cow 516, general condition fairly good, had been affected with tuberculosis eighteen months or longer. Microscopic examinations of feces on seven different days revealed tubercle bacilli on four days.

On February 1, 1909, two guinea pigs, Nos. 2831 and 2832, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 13, 1909 (seventy-one days after injection), and on autopsy were found to be free from lesions of disease.

Cow 536, general condition poor, had been affected with tuberculosis two years or longer. Microscopic examinations of feces on eleven different days revealed tubercle bacilli on eight days.

On February 3, 1909, two guinea pigs, Nos. 2865 and 2866, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 13, 1909 (sixty-nine days after injection), and on autopsy were found to be free from lesions of disease.

Cow 551, general condition fairly good, had been affected with tuberculosis two years or longer. Microscopic examinations of feces on nine different days revealed tubercle bacilli on three days.

On February 1, 1909, two guinea pigs, Nos. 2833 and 2834, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 12, 1909 (seventy days after injection), and on autopsy were found to be free from lesions of disease.

Cow 553, general condition fairly good, had been affected with tuberculosis two years or longer. Microscopic examinations of feces on three different days revealed tubercle bacilli every day.

On February 1, 1909, two guinea pigs, Nos. 2827 and 2828, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 13, 1909 (seventy-one days after injection), and on autopsy were found to be free from lesions of disease.

Cow 620, general condition good, had been affected with tuberculosis a year or longer. Microscopic examinations of feces on four different days revealed tubercle bacilli on one day.

On February 3, 1909, two guinea pigs, Nos. 2855 and 2856, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 13, 1909 (sixty-nine days after injection), and on autopsy were found to be free from lesions of disease.

Cow 629, general condition fair, had been affected with tuberculosis at least one year. Microscopic examinations of feces on five different days revealed tubercle bacilli on two days.

On February 3, 1909, two guinea pigs, Nos. 2857 and 2858, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 13, 1909 (sixty-nine days after injection), and on autopsy were found to be free from lesions of disease.

Cow 631, general condition fair, had been affected with tuberculosis at least one year. Microscopic examinations of feces on four different days revealed tubercle bacilli on one day.

On February 4, 1909, two guinea pigs, Nos. 2877 and 2878, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 13, 1909 (sixty-eight days after injection), and on autopsy were found to be free from lesions of disease.

Bull 635, general condition good, had been affected with tuberculosis over two years. Microscopic examinations of feces on three different days revealed tubercle bacilli on one day.

On January 20, 1909, two guinea pigs, Nos. 2817 and 2818, received each an intraabdominal injection of 3 c. c. of blood from the bull. Guinea pig 2818 died prematurely as a result of the injection. Guinea pig 2817 was killed April 12, 1909 (seventy-two days after injection), and on autopsy was found to be free from lesions of disease.

Cow 636, general condition fairly good, had been affected with tuberculosis at least one year. Microscopic examinations of feces on five different days revealed tubercle bacilli on two days.

On January 30, 1909, two guinea pigs, Nos. 2803 and 2804, received each an intraabdominal injection of 3 c. c. of blood from the cow. The guinea pigs were killed April 12, 1909 (seventy-two days after injection), and on autopsy were found to be free from lesions of disease.

Cow 638, general condition fairly good, had been affected with tuberculosis over two years. Microscopic examinations of feces on four different days revealed tubercle bacilli on all four days.

Guinea pigs were given intraabdominal injections of the blood of this cow as follows:

January 29, 1909, guinea pig 2795 received 5 c. c.

January 29, 1909, guinea pig 2796 received 5 c. c.

February 4, 1909, guinea pig 2879, received 3 c. c.

February 4, 1909, guinea pig 2880 received 3 c. c.

Guinea pig 2795 died prematurely as a result of the injection. Guinea pig 2796 was killed April 12, 1909 (seventy-three days after the injection), and on autopsy was found to be free from lesions of disease. Guinea pigs 2879 and 2880 were killed April 12, 1909 (sixty-seven days after injection), and on autopsy were found to be free from lesions of disease.

Cow 639, general condition good, had been affected with tuberculosis at least one year. Microscopic examinations of feces on four different days revealed tubercle bacilli on one day.

Guinea pigs were given intraabdominal injections of the blood of this cow as follows:

January 29, 1909, guinea pig 2801 received 5 c. c.

January 29, 1909, guinea pig 2802 received 5 c. c.

February 5, 1909, guinea pig 2887 received 3 c. c.

February 5, 1909, guinea pig 2888 received 3 c. c.

Guinea pig 2801 died prematurely as a result of the injection. Guinea pig 2802 was killed April 12, 1909 (seventy-three days after injection), and on autopsy several small necrotic foci were found in the liver and spleen. The lesions were not at all like the conditions caused by the tubercle bacillus, and microscopic examinations failed to reveal tubercle bacilli. Some of the abnormal tissue was used to make subinoculations into guinea pigs: the subinoculated guinea pigs failed to show tuberculosis.

Guinea pigs 2887 and 2888 were killed April 13, 1909 (sixty-seven days after the injection), and on autopsy were found to be free from lesions of disease.

Cow 640, general condition good, had been affected with tuberculosis over two years. Microscopic examinations of feces on three different days revealed tubercle bacilli on all three days.

On January 29, 1909, two guinea pigs, Nos. 2799 and 2800, received each an intraabdominal injection of 5 c. c. of blood from the cow. The guinea pigs were killed April 12 1909 (seventy-three days after injection), and on autopsy were found to be free from lesions of disease.

Cow 642, general condition good, had been affected with tuberculosis at least one year. Microscopic examinations of feces on three different days revealed tubercle bacilli on two days.

Guinea pigs were injected intraabdominally with blood from this cow as follows:

January 29, 1909, guinea pig 2793 received 5 c. c.

January 29, 1909, guinea pig 2794 received 5 c. c.

February 5, 1909, guinea pig 2885 received 3 c. c.

February 5, 1909, guinea pig 2886 received 3 c. c.

Guinea pig 2794 died prematurely as the result of the injection. Guinea pig 2886 died March 5, 1909 (twenty-eight days after injection), of an intercurrent affection, and the autopsy revealed no lesions resembling tuberculosis. Guinea pig 2793 was killed April 13, 1909 (seventy-three days after injection), and on autopsy was found to be free from lesions of disease. Guinea pig 2885 was killed April 13, 1909 (sixty-seven days after injection), and on autopsy was found to be free from lesions of disease.

LOT 4.

Cow 479, general condition fair, but had greatly enlarged throat glands, had been affected with tuberculosis about three years.

On February 4, 1909, two guinea pigs, Nos. 2873 and 2874, received each an intraabdominal injection of 3 c. c. of her blood. The guinea pigs were killed April 13, 1909 (sixty-eight days after injection), and on autopsy were found to be free from lesions of disease.

Bull 508, general condition good, had been affected with tuberculosis about two and one-half years.

On February 4, 1909, two guinea pigs, Nos. 2867 and 2868, received each an intraabdominal injection of 3 c. c. of his blood. The guinea pigs were killed April 12, 1909 (sixty-seven days after injection), and on autopsy were found to be free from lesions of disease.

Cow 517, general condition good, had been affected with tuberculosis about two and one-half years.

On February 1, 1900, two guinea pigs, Nos. 2825 and 2826, received each an intraabdominal injection of 3 c. c. of her blood. Guinea pig 2825 died of an intercurrent affection April 5, 1900 (sixty-three days after injection), and on autopsy was found to be free from lesions of tuberculosis. Guinea pig 2826 was killed April 13, 1900 (seventy-one days after injection), and on autopsy was found to be free from lesions of disease.

Cow 569, general condition fair, had been affected with tuberculosis about two years.

On February 1, 1900, two guinea pigs, Nos. 2819 and 2820, received each an intraabdominal injection of 3 c. c. of her blood. The guinea pigs were killed April 12, 1900 (seventy days after injection), and on autopsy were found to be free from lesions of disease.

Cow 630, general condition fair, had been affected with tuberculosis about one year.

On February 3, 1900, two guinea pigs, Nos. 2853 and 2854, received each an intraabdominal injection of 3 c. c. of her blood. The guinea pigs were killed April 13, 1900 (sixty-nine days after injection), and on autopsy were found to be free from lesions of disease.

Cow 632, general condition good, had been affected with tuberculosis at least one year.

On January 30, 1900, two guinea pigs, Nos. 2807 and 2808, received each an intraabdominal injection of 3 c. c. of her blood. Guinea pig 2808 died prematurely as a result of the injection. Guinea pig 2807 was killed April 13, 1900 (seventy-two days after injection), and on autopsy was found to be free from lesions of disease.

Cow 633, general condition fair, had been affected with tuberculosis at least one year.

On January 30, 1900, two guinea pigs, Nos. 2813 and 2814, received each an intraabdominal injection of 3 c. c. of her blood. Guinea pig 2813 died prematurely as a result of the injection. Guinea pig 2814 was killed on April 12, 1900 (seventy-one days after injection), and on autopsy was found to be free from lesions of disease.

Cow 634, general condition fair, had been affected with tuberculosis at least one year.

On January 20, 1900, two guinea pigs, Nos. 2787 and 2788, received each an intraabdominal injection of 3 c. c. of her blood. The guinea pigs were killed April 12, 1900 (seventy-three days after injection), and on autopsy were found to be free from lesions of disease.

Cow 641, general condition fair, had been affected with tuberculosis over two years.

On January 29, 1900, two guinea pigs, Nos. 2797 and 2798, received each an intraabdominal injection of 5 c. c. of the blood of the cow, and on February 4, 1900, two guinea pigs, Nos. 2875 and 2876, received each a similar injection of 3 c. c. of blood. Guinea pig 2798 died prematurely as a result of the injection. Guinea pig 2797 was killed April 12, 1900 (seventy-five days after injection), and guinea pigs 2875 and 2876 were killed April 12, 1900 (sixty-seven days after injection). The last three guinea pigs were found to be free from lesions of disease.

Cow 644, general condition fair, had been affected with tuberculosis four months or more.

On February 2, 1900, two guinea pigs, Nos. 2849 and 2850, received each an intraabdominal injection of 3 c. c. of blood of the cow. The guinea pigs were killed April 13, 1900 (seventy days after injection), and on autopsy were found to be free from lesions of disease.

Cow 645, general condition good, had been affected with tuberculosis three months or more.

On February 2, 1909, two guinea pigs, Nos. 2845 and 2846, received each an intraabdominal injection of 3 c. c. of her blood. Guinea pig 2845 died prematurely as a result of the injection. Guinea pig 2846 was killed April 13, 1909 (seventy days after injection), and on autopsy was found to be free from lesions of disease.

Cow 648, general condition good, had been affected with tuberculosis for an unknown period of time. She was brought to the Experiment Station shortly before her blood was used for guinea-pig injections, and reacted with tuberculin.

On February 2, 1909, two guinea pigs, Nos. 2837 and 2838, received each an intraabdominal injection of 3 c. c. of her blood. The guinea pigs were killed April 12, 1909 (sixty-nine days after injection), and on autopsy were found to be free from lesions of disease.

Cow 657, general condition fair, had been affected with tuberculosis for an unknown period of time. She was brought to the Experiment Station shortly before her blood was used for guinea-pig injections, and reacted with tuberculin.

On February 2, 1909, two guinea pigs, Nos. 2841 and 2842, received each an intraabdominal injection of 3 c. c. of her blood. The guinea pigs were killed April 12, 1909 (sixty-nine days after injection), and on autopsy were found to be free from lesions of disease.

DISCUSSION OF RESULTS.

Among the 42 cattle enumerated above, 27, or 64½ per cent, were shown by microscopic examinations to be discharging tubercle bacilli from their bowels—in most instances intermittently—and the infectious character of the feces in 7 cases, or 16⅔ per cent, was demonstrated by animal experiments—that is, feeding and inoculation tests.

These two facts—that 27 of the cattle were shown by microscopic tests to be expelling tubercle bacilli per rectum, while only 7 were proved by animal experiments to be passing infected feces—must not be taken as being in any sense contradictory, as the feces of only a sufficient number of tuberculous cattle were tested by animal feeding and inoculation experiments to prove conclusively that the acid-fast bacilli found on microscopic examinations in the feces of tuberculous cattle are certainly live, virulent tubercle bacilli.

Relative to the expulsion of tubercle bacilli from the bowels of tuberculous cattle, all the evidence we have indicates that the bacilli have their origin in the lung and throat, from which regions they are coughed up, swallowed, and passed through and out of the intestinal canal without appreciable loss of pathogenic virulence. That a large proportion of the tubercle bacilli swallowed by cattle really pass through their bodies and out per rectum without a determinable loss of virulence was experimentally shown in some of our earlier work.^a We have absolutely no reason to believe that tubercle bacilli

^a Bureau of Animal Industry, Bulletins 88 and 99.

enter the intestinal canal from the lymph radicals or blood capillaries or by any complex and mysterious system of transportation from lesions of all descriptions and kinds in any or every portion of the body. It is our conviction that, unless an open tuberculosis is in more or less direct communication with the intestinal canal or there is a tuberculous disease of the intestine itself, which latter is rare among cattle, no tubercle bacilli will be expelled with the feces.

If tuberculosis in all its forms was a bacteriemia the expulsion of tubercle bacilli from the bowels of all tuberculous individuals, as well as with their urine, saliva, milk, and other bodily secretions, would follow as a natural consequence. Those who have carefully studied the secretions from the uninvolved organs of tuberculous subjects know how rarely tubercle bacilli are detected in them even with the application of the most delicate tests.

When we consider cattle like Nos. 533, 549, and 552—three of the four animals of which autopsy records are given—and note that they were so badly diseased that they would have been condemned on superficial examination as wholly unfit for use as food under the existing meat-inspection regulations, the absence of tubercle bacilli from their blood may be regarded as a sufficient reason for assuming that the possible occurrence of tubercle bacilli in the blood of tuberculous animals will almost invariably be associated with pathological conditions of a very marked character, or that the tubercle bacilli will be present in extremely small numbers and will speedily be filtered out of the blood stream. Cow 533 had been affected with tuberculosis two years or longer, was in poor condition as a result of the disease, and on autopsy was found to have an extensive, open tuberculosis of the lung and lesions of tuberculosis in the liver and in both the thoracic and abdominal lymph glands. Cow 549 was, if anything, even more severely and extensively affected, and had given birth to a congenitally tuberculous calf less than a year before her blood was injected into guinea pigs. Cow 552 was also affected with generalized, advanced, open tuberculosis, and prior to the use of her blood for the guinea-pig injections was found to be passing from her bowels large numbers of tubercle bacilli, which were proved by feeding tests to be virulent for hogs and by inoculation tests to be virulent for guinea pigs. With the blood obtained from these three cows 14 guinea pigs were injected, of which 2 died prematurely and 12 lived two months or more afterwards, until they were intentionally killed, when they were found on post-mortem examination to be wholly free from lesions of disease of any kind.

The possibility exists that tubercle bacilli introduced into the stomach and intestine by swallowing may be taken up by the lymph radicals, passed along the lymph channels, and emptied through the

great lymph ducts into the venous circulation. The investigations of Nicolas and Descos, Ravenel, Calmette and Guérin, Schlossman and Engle, and others speak for this; but such tubercle bacilli will not be very numerous and will no doubt be filtered out of the blood as soon as it reaches the lung through the heart and pulmonary arteries, to which it passes directly after it has received the lymph stream.

SUPPLEMENTAL TESTS REGARDING POSSIBLE IMMUNITY.

We have already stated that the possibility exists that the intra-peritoneal injection of from 3 to 5 c. c. of fresh, warm blood from tuberculous cattle induces an immunity in guinea pigs to the tubercle bacilli the blood may contain. Although we knew of nothing to uphold this theory, we considered it necessary to undertake an investigation to prove or disprove it, the results of which are now presented.

On April 24, 1909, blood and tuberculous material was obtained from cow 533 (see record of cow on p. 11) for a number of guinea-pig injections. The primary object of the injections was to prove that the blood of a tuberculous cow, when introduced into the peritoneal cavity of a guinea pig, has no retarding influence on the development of tuberculosis from tubercle bacilli that may be present in it.

Cow 533 was first bled from the jugular vein and then at once killed. As soon as she was dead a tuberculous mediastinal gland was removed from her body and 500 mg. of it emulsified with 2 c. c. of sterile, normal salt solution. Cover glasses of this emulsion, stained with carbolfuchsin and decolorized with 20 per cent sulphuric acid, revealed on microscopic examination, on an average, two tubercle bacilli each. The emulsion was mixed with an additional quantity of sterile, normal salt solution, so that each cubic centimeter of the dilution represented a strength equal to one drop of the original emulsion.

The blood obtained from the cow prior to her death and the diluted emulsion made with the tuberculous mediastinal gland from her body were used to inject seven groups of guinea pigs, the records of which are given below.

The guinea pigs in the seven different groups were injected for the following purposes: Group 1, to serve as checks on the absence or presence of tubercle bacilli in the blood of the tuberculous cow that was used for the investigation; group 2, to show that the intra-abdominal injection of fresh, warm blood from a tuberculous cow can not protect against tubercle bacilli simultaneously introduced into the abdominal cavity; group 3, to show that the intraabdominal injection of fresh, warm blood from a tuberculous cow can not protect against tubercle bacilli introduced into other parts of the body than the abdominal cavity; groups 4 and 5, to show that the blood of

tuberculous cows has no special germicidal potency for tubercle bacilli; groups 6 and 7, to serve as guides relative to the amount of tuberculous disease to be expected in the bodies of the guinea pigs that were injected with both blood and emulsion of tuberculous material.

GROUP 1.

On April 24, 1909, eight guinea pigs, Nos. 3626 to 3633, inclusive, received each an intraabdominal injection of 3 c. c. of freshly drawn warm blood. On May 6, 1909, guinea pig 3627 died affected with congestion of the lungs. On autopsy no lesions of tuberculosis were found. On May 27 and 28, 1909, guinea pigs 3626, 3628, 3629, 3630, 3631, 3632, and 3633 were killed and examined post-mortem. No lesions of tuberculosis or other disease were found.

GROUP 2.

On April 24, 1909, eight guinea pigs, Nos. 3642 to 3649, inclusive, received each an intraabdominal injection of 3 c. c. of freshly drawn blood, followed as quickly as possible by an intraabdominal injection of 0.5 c. c. of tuberculous emulsion. On April 30, 1909, guinea pig 3642 died affected with inflammation of the large bowel. On May 27, 1909, guinea pigs 3643, 3644, 3645, 3646, 3647, 3648, and 3649 were killed and examined post-mortem. Every one of the seven was found to be affected with generalized tuberculosis of the abdominal and thoracic organs.

GROUP 3.

On April 24, 1909, eight guinea pigs, Nos. 3634 to 3641, inclusive, received each an intraabdominal injection of freshly drawn warm blood, followed as soon as possible by a subcutaneous injection into the right thigh of 0.5 c. c. of tuberculous emulsion. On May 27, 1909, guinea pigs 3636 and 3637 and on May 28, 1909, guinea pigs 3634, 3635, 3638, 3639, 3640, and 3641 were killed and examined post-mortem. The eight guinea pigs all showed more or less extensive lesions of tuberculosis at the seat of the subcutaneous injection, tuberculosis of the adjacent superficial inguinal gland, tuberculosis of the pelvic, lumbar, and gastro-hepatic glands, and a sprinkling of tuberculous foci in the liver and spleen.

GROUP 4.

On April 24, 1909, eight guinea pigs, Nos. 3658 to 3665, inclusive, received each an intraabdominal injection of 3 c. c. of a mixture of defibrinated blood and tuberculous emulsion. Each 3 c. c. of this mixture was equivalent to 0.5 c. c. of the diluted tuberculous emulsion described earlier. The mixture was two hours old at the time it was injected into the guinea pigs. On April 25, 1909, guinea pigs 3659, 3660, and 3661 died. The post-mortem examinations showed no lesions excepting a congested condition of the lungs and a quantity of unabsorbed blood in the peritoneal cavity. On May 12, 1909, guinea pig 3662 died affected with congestion of the lungs; no lesions of tuberculosis were found. On May 27, 1909, guinea pigs 3658, 3663, 3664, and 3665 were killed and examined post-mortem. Three of them were affected with completely generalized tuberculosis of the abdominal and thoracic organs, and the remaining one (No. 3665) with generalized tuberculosis of the abdominal organs only.

GROUP 5.

On April 26, 1909, eight guinea pigs, Nos. 3670 to 3677, inclusive, received each an intraabdominal injection of 3 c. c. of the same mixture of blood and

tuberculous emulsion used for the guinea pigs of group 4. The mixture was forty-five hours old at the time it was injected. On May 27, 1909, the eight guinea pigs were killed and examined post-mortem. Seven of them were affected with generalized tuberculosis of the abdominal and thoracic organs, and one (No. 3672) with generalized tuberculosis of the abdominal organs only.

GROUP 6.

On April 24, 1909, four guinea pigs, Nos. 3650 to 3653, inclusive, received each an intraabdominal injection of 0.5 c. c. of tuberculous emulsion. On May 6, 1909, guinea pig 3651 died affected with congestion of the lungs. On May 27, 1909, Nos. 3650, 3652, and 3653 were killed and examined post-mortem. No. 3650 showed tuberculous lesions of the spleen and omentum only, and Nos. 3652 and 3653 showed a fairly generalized tuberculosis of the abdominal and thoracic organs.

GROUP 7.

On April 24, 1909, four guinea pigs, Nos 3654 to 3657, inclusive, received each a subcutaneous injection, in the right thigh, of 0.5 c. c. of tuberculous emulsion. On May 27, 1909, the four guinea pigs were killed and examined post-mortem. Nos. 3654 and 3656 each showed a small tuberculous abscess at the seat of injection, a tuberculous condition of the superficial inguinal gland near the seat of injection, and a fairly generalized tuberculosis of the pelvic and abdominal organs. Guinea pigs 3655 and 3657 showed similar lesions with the exception of the abscesses at the seat of injection.

The autopsy records of the guinea pigs show, in a general way, very little difference between the animals that received only tuberculous emulsion and those that received both blood and emulsion. The guinea pigs that received both blood and emulsion into their abdominal cavities showed numerically more extensive lesions of tuberculosis than the guinea pigs that received only emulsion into their abdominal cavities. This condition would naturally be expected because the same number of tubercle bacilli contained in 3 c. c. of blood would be more widely separated and in better condition to start a large number of individual lesions than those in 0.5 c. c. of salt solution.

The use of an emulsion of tuberculous tissue from the tuberculous cow that supplied the blood for the supplemental injections was preferred to the use of a pure culture of tubercle bacilli, because it seemed desirable to us to use infectious material and blood in this instance from the same individual case of tuberculosis.

The total number of guinea pigs injected in this supplemental investigation was 48, of which 8 received blood only, 32 both blood and tuberculous material, and 8 tuberculous material only. Of the 32 that received both blood and tuberculous material and the 8 that received only tuberculous material, 6 died prematurely, and the remaining 34, when they were killed—thirty to thirty-one days after the injection—were all found to be affected with tuberculosis of a form that would have progressed to death in a short time.

Among the 8 guinea pigs that received an injection of fresh warm blood without the addition of tuberculous material, 1 died prematurely and the remaining 7 were found on autopsy to be free from lesions of disease. Since the cow that supplied the blood for the injections was affected, as her record shows, with extensive, advanced tuberculosis, the 7 guinea pigs make a strong addition to the 88 parallel cases of which the records have been previously given; and hence we have 95 guinea pigs as the total number that received injections of blood from tuberculous cattle into their peritoneal cavities—the most delicate test for tubercle bacilli available—and survived the injection long enough for tuberculosis to manifest itself clearly. Among this total of 95 guinea pigs not one case of tuberculosis developed.*

CONCLUSIONS.

1. We failed utterly to find tubercle bacilli in the blood of tuberculous cattle which we examined microscopically in accordance with the method described and used by Doctor Rosenberger.

2. The negative results of our microscopic examinations are confirmed by the negative results obtained with 95 guinea pigs, each of which received an intraabdominal injection of blood from a tuberculous cow or bull.

3. As the number of cattle from which blood was injected into the 95 guinea pigs was 42, and as these cattle represented practically all stages of tuberculosis from mildly affected recent cases to old and completely generalized cases, we feel that our work shows beyond the remotest doubt that tuberculosis is not to be classified, in any sense of the word, as a bacteriemia.

* An independent investigation relative to the occurrence of tubercle bacilli in the circulating blood of cattle was made in the Bureau of Animal Industry by Dr. John R. Mohler, chief of the Pathological Division. Mohler examined the blood of 8 cattle microscopically, and with blood from each of these cattle injected 5 guinea pigs. The microscopic examinations and injections were made precisely in the manner described by Doctor Rosenberger. No tubercle bacilli were discovered microscopically, and not one of the 40 injected guinea pigs contracted tuberculosis. Two of the 8 cattle were in good condition, but were passing tubercle bacilli from their bowels; 2 of the cattle were in poor condition and were passing tubercle bacilli from their bowels; and 4 of the cattle were slaughtered for meat, but on inspection were found to be so extensively affected with tuberculosis that it was necessary to condemn and tank their carcasses under the Federal meat-inspection regulations. This evidence, kindly presented to us by Doctor Mohler, raises the number of tuberculous cattle from which blood was tested to 50, and the number of guinea pigs that received injections of blood from tuberculous cattle without contracting tuberculosis to 135

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U. S. DEPARTMENT OF AGRICULTURE,

BUREAU OF ANIMAL INDUSTRY.—BULLETIN 117.

A. D. MELVIN, CHIEF OF BUREAU.

LEUCOCYTES IN MILK:

METHODS OF DETERMINATION AND THE
EFFECT OF HEAT UPON THEIR NUMBER.

BY

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Expert in Milk Hygiene, Pathological Division.

(In cooperation with the Pennsylvania State Live-Stock Sanitary Board.)



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LETTER OF TRANSMITTAL.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY,
Washington, D. C., July 12, 1909.

SIR: I have the honor to transmit herewith and to recommend for publication as a bulletin of this Bureau a manuscript entitled "Cultural Studies of Species of *Penicillium*," by Dr. Charles Thom, mycologist in the cooperative soft-cheese investigations carried on at Storrs, Conn., by the Storrs Agricultural Experiment Station and the Dairy Division of this Bureau. A previous paper of this author dealing with certain species of *Penicillium* which are characteristic factors in the ripening of Camembert and Roquefort cheese has been published as Bulletin 82 of this Bureau. The investigations connected with the study of the organisms referred to necessitated the culture and comparison of many other species and an examination of the nomenclature for the whole genus *Penicillium*. Considerable confusion was found to exist regarding the identification of the various species, hence it seemed important that a comprehensive study from cultural data of all obtainable species should be undertaken.

Acknowledgment is made of suggestions and advice by Dr. Erwin F. Smith, of the Bureau of Plant Industry of this Department, besides the persons whose names are given in the text.

The illustrations, with one exception, are from original drawings by the author.

Respectfully,

Hon. JAMES WILSON,
Secretary of Agriculture.

A. D. MELVIN,
Chief of Bureau.

CONTENTS.

	Page.
Introduction.....	9
The basis for specific characterization.....	10
Necessity for describing culture media and temperature conditions.....	14
Habit, structure, and appearance of colonies.....	15
Physiological effects upon media.....	19
Culture media.....	22
Nomenclature.....	23
The generic name.....	23
Nomenclature of species.....	23
The type species.....	25
Explanation of drawings.....	27
<i>Penicillium expansum</i> Link.....	27
<i>Penicillium italicum</i> Wehmer.....	29
<i>Penicillium digitatum</i> Saccardo.....	31
<i>Penicillium roqueforti</i> Thom.....	34
<i>Penicillium purpurogenum</i> O. Stoll.....	36
<i>Penicillium pinophilum</i> Hedgcock (nomen novum).....	37
<i>Penicillium rubrum</i> O. Stoll.....	39
<i>Penicillium luteum</i> Zukal.....	39
<i>Penicillium duclauxi</i> Delacroix.....	42
<i>Penicillium claviforme</i> Bainier.....	43
<i>Penicillium granulatum</i> Bainier.....	44
<i>Penicillium brevicaulis</i> Saccardo.....	45
<i>Penicillium brevicaulis</i> Saccardo, var. <i>album</i> Thom, n. var.....	47
<i>Penicillium brevicaulis</i> Saccardo, var. <i>glabrum</i> Thom, n. var.....	48
<i>Penicillium roseum</i> Link (?).....	49
<i>Penicillium camemberti</i> Thom.....	50
<i>Penicillium camemberti</i> var. <i>rogeri</i> Thom, n. var.....	52
<i>Penicillium biforme</i> n. sp.....	54
<i>Penicillium commune</i> n. sp.....	56
<i>Penicillium</i> No. 22.....	58
<i>Penicillium chrysogenum</i> n. sp.....	58
<i>Penicillium rugulosum</i> n. sp.....	60
<i>Penicillium citrinum</i> n. sp.....	61
<i>Penicillium</i> No. 37.....	63
<i>Penicillium</i> No. 12.....	64
<i>Penicillium atramentosum</i> n. sp.....	65
<i>Penicillium</i> No. 24.....	66
<i>Penicillium stoloniferum</i> n. sp.....	68
<i>Penicillium funiculosum</i> n. sp.....	69
<i>Penicillium decumbens</i> n. sp.....	71
<i>Penicillium divaricatum</i> n. sp.....	72
<i>Penicillium lilacinum</i> n. sp.....	73

	Page.
<i>Penicillium intricatum</i> n. sp.....	75
<i>Penicillium spinulosum</i> n. sp.....	76
<i>Penicillium</i> No. 28.....	77
Species forming pink sclerotia.....	78
<i>Penicillium</i> No. 29.....	78
<i>Penicillium</i> No. 30.....	79
<i>Penicillium</i> No. 31.....	80
<i>Penicillium</i> No. 32.....	80
Comparative cultural data.....	81
Cultures in distilled water.....	82
Agar-agar as a source of food.....	83
Agar-agar as a source of carbon.....	83
Various sources of carbon.....	83
Cultures in Raulin's fluid and in Cohn's solution.....	86
Color in conidial areas.....	87
Effect of concentrated media.....	88
The grouping of species.....	89
Odors.....	90
Anaerobic cultures (with carbon dioxid).....	90
Incubation tests.....	91
Summary of data from comparative culture.....	94
Keys to cultural identification of species.....	94
KEY 1.—Analysis of species in cultures upon gelatin and agar.....	95
KEY 2.—Species determinable from substrata.....	97
Tabular statements.....	98
TABLE 1.—Tabulation of salient characters of species.....	98
TABLE 2.—Gelatin and color reactions.....	100
TABLE 3.—Comparative cultures.....	101
TABLE 4.—Comparative cultures in synthetic fluid (Dox's).....	102
TABLE 5.—Decimal summary of comparative cultures in synthetic fluid (Dox's).....	104
TABLE 6.—Incubation experiments.....	105
References to literature.....	106
Index to species.....	109

ILLUSTRATIONS.

	Page.
FIG. 1. <i>Penicillium expansum</i> Link.....	28
2. <i>Penicillium italicum</i> Wehmer.....	30
3. <i>Penicillium digitatum</i> Saccardo.....	33
4. <i>Penicillium roqueforti</i> Thom.....	35
5. <i>Penicillium purpurogenum</i> O. Stoll.....	37
6. <i>Penicillium pinophilum</i> Hedgcock.....	38
7. <i>Penicillium rubrum</i> O. Stoll.....	40
8. <i>Penicillium luteum</i> Zukal.....	41
9. <i>Penicillium duclauxi</i> Delacroix.....	42
10. <i>Penicillium claviforme</i> Bainier.....	43
11. <i>Penicillium granulatum</i> Bainier.....	45
12. <i>Penicillium brevicaulis</i> Saccardo.....	46
13. <i>Penicillium brevicaulis</i> , var. <i>album</i>	47
14. <i>Penicillium brevicaulis</i> , var. <i>glabrum</i>	48
15. <i>Penicillium roseum</i> Link (?).....	49
16. <i>Penicillium camemberti</i> Thom.....	51
17. <i>Penicillium camemberti</i> , var. <i>rogeri</i>	53
18. <i>Penicillium biforme</i>	55
19. <i>Penicillium commune</i>	57
20. <i>Penicillium chrysogenum</i>	59
21. <i>Penicillium rugulosum</i>	61
22. <i>Penicillium citrinum</i>	62
23. <i>Penicillium</i> No. 37.....	64
24. <i>Penicillium atramentosum</i>	66
25. <i>Penicillium</i> No. 24.....	67
26. <i>Penicillium stoloniferum</i>	68
27. <i>Penicillium funiculosum</i>	70
28. <i>Penicillium decumbens</i>	71
29. <i>Penicillium divaricatum</i>	73
30. <i>Penicillium lilacinum</i>	74
31. <i>Penicillium intracatum</i>	75
32. <i>Penicillium spinulosum</i>	76
33. <i>Penicillium</i> No. 28.....	77
34. <i>Penicillium</i> No. 29.....	79
35. <i>Penicillium</i> No. 31.....	80
36. <i>Penicillium</i> No. 32.....	81

CULTURAL STUDIES OF SPECIES OF *PENICILLIUM*.

INTRODUCTION.

In a previous paper ^{25a} two species of *Penicillium* were shown to secrete the proteolytic enzymes which ripen certain varieties of cheese. The effort to identify these organisms necessitated the culture and comparison of numerous other species and a study of the literature of nomenclature for the whole of the genus *Penicillium*. The difficulties encountered in deciding whether to discuss these forms under old names or to describe them anew from cultural data led to the extension of this study beyond the forms occurring in a dairy investigation so as to include any obtainable species, and especially all species whose identification under published names could be established.

Many recent studies have linked particular chemical and physiological activities with the presence of particular species of fungi. When such data relate to known species—identifiable species—our knowledge of the metabolism of these forms has been greatly increased. When, as has often happened, the generic name alone is given in a group so diverse in its activities as *Penicillium*, such data only add to the confusion. Whether the observations apply to all of the species of the genus or to a single unnamed species is left unsettled. To give real utility to such work, some one must test the applicability of the data to each species under consideration. On account of their ease of cultivation and the notion that there was a single common green species legitimately named *P. glaucum*, species of *Penicillium* have formed the subjects of many such investigations. In very few of these cases are sufficient data regarding morphology given to warrant even a guess as to the species used. An examination of the present status of specific nomenclature in the genus will therefore furnish a sounder basis for further studies of their activities.

This paper represents cultural work which has continued more than four years and includes those species for which the data obtained seem abundantly to justify the characterization offered. Some of these forms have been cultivated for the whole period, others for a less time. No claim to monographic completeness can be made. In

^a The figures refer to the list of literature at end of bulletin.

collecting these forms, many of them have been isolated in this laboratory from dairy products and from fruits, or collected in the field by the writer. One series of forms was purchased from Kral, of Prague. Several species have been contributed by or verified by those who described them—two by Dr. G. Bainier, two by Dr. C. Wehmer, one by Dr. G. Delacroix, and one by Dr. G. G. Hedgcock. Many correspondents have sent cultures for examination or study.

THE BASIS FOR SPECIFIC CHARACTERIZATION.

The available sources of identification of these species will be discussed first. By the kindness of Professor Thaxter the exsiccati of the genus in the herbarium of Harvard University were examined. Cultures of certain species were tried but no living spores were found. No specimen was found in such condition as to be used to identify material by comparison. Similar courtesy was extended for the examination of specimens in the herbarium of Kew Gardens and at the University of Berlin. In no case was material found by which cultural material could be identified or identification verified by comparison. The plant bodies are too evanescent to retain the criteria necessary for identification for any great length of time, as ordinarily preserved. In most cases after a few years of handling the specimens were found reduced to powder. Wehmer²² has noted for certain species of this genus that the conidia do not retain their power to germinate beyond a very few years. Specimens as formerly prepared therefore become useless for the identification of species by the method of types. The method described by Hedgcock⁹ for preserving specimens has thus far been applied only to one species of *Penicillium* and entirely too recently to test its permanent value. Aside, therefore, from certain species which will be discussed later, it has been impossible to determine material belonging to this genus from herbarium specimens.

There remain two methods of identification: (1) The identification of material or cultures by the authors of descriptions; (2) identification by critical study of the descriptions and illustrations published.

Regarding the first method, fortunately some of the more recent authors have either preserved their organisms in culture, as is done at the Ecole de Pharmacie in Paris with the cultures of Bainier, or placed them in one other of the distributing laboratories where such organisms are maintained in continuous culture. But the earlier authors are dead and have left only their published descriptions and figures as a means of determining what organisms they studied.

Turning to the critical comparison of material with published descriptions, we find many difficulties. These descriptions give in meager outline observations of mold masses showing penicillate conidial fructifications, as found in nature upon substrata more or less

accurately specified. No cultures were made. The original masses are assumed to be comprised each of a single species. Parasitism or selective saprophytism is assumed, but the substratum is rarely designated with sufficient care to make a duplication of the original culture possible. Hence the characters would include whole series of forms whose differences are marked. No account is taken of their omnivorous nature nor of the marked variations introduced in appearance by changed conditions. The method of types and the method of substrata as represented by the herbarium material and the published descriptions we have are, therefore, not sufficient for the identification of species of *Penicillium*.

Although we abandon the "method of substrata" as the sole basis of description, the force of natural selection as shown by the distribution of certain of these species in nature is a most valuable accessory. Some species occur so constantly upon particular substrata and under particular conditions as to simplify their identification greatly. Examples of this are *P. italicum* Wehmer and *P. digitatum* Saccardo, as they are found upon citrus fruits. Unfortunately very few species restrict themselves to particular substrata, so that except for some few species and a very few media, the occurrence of a *Penicillium* in any given situation is but slight evidence for its identification. The constant occurrence of species of *Penicillium* in the laboratory, in connection with foods, and in factory processes, such as cheese-ripening, all point to controlled culture as the proper source of diagnostic characters.

If we look to cultural study^a for our conception of species we have two methods of procedure: (1) The exhaustive study of the limits of variability for each species; (2) the comparative study of numerous species under arbitrarily chosen conditions uniformly maintained.

The first is the best method known for gaining complete knowledge of single species, but it is too cumbrous for taxonomic purposes. Physiological and chemical studies have commonly been restricted to particular classes of reactions for single species or groups of species. These have contributed much to our knowledge of fungous variability, but too often give no hint, except the vague nomenclature used, upon which to judge which species were actually studied. In experimental cultures changes in the chemical nature of the medium or in the conditions, or both, have been found to produce great changes in the morphology of the fungi studied. With the exception of a few fundamental group or generic characters, nearly every

^aWeidemann,³⁵ in a recent paper, has described several new species of this genus in their relation to culture media. In this he has followed the bacteriologists further than the writer has thought necessary by giving formulas for a considerable number of media and detailed notes as to reactions upon such media, instead of the more formal descriptions attempted here.

attribute used in specific description has been shown to be a reaction to environment, hence changeable with such environment (for some species at least). Exhaustive study of all the species would be endless.

The alternative is to select certain media and a particular set of conditions, then to cultivate all the organisms under investigation in a uniform manner and to base distinctions of species upon differences in the reactions obtained, and upon the differing characters of the several species, in this common environment.

To test the reliability of such data, species obtained in the dairy laboratories were first studied carefully upon the peptone-milk-sugar gelatin described by Conn⁴ and upon potato agar as described in our previous bulletin (Thom,²⁵ p. 7). From these cultures transfers have been made to media of very different composition—Cohn's solution, Raulin's fluid, milk in various forms, synthetic fluids presenting different sources of carbon—always bringing cultures back to the original media. Many species differ so materially in gross characters when grown upon these different media that successive cultures, if not known to be pure transfers, might be supposed to be different species; but when returned to the original media and conditions these forms have immediately produced the characters and reactions first found, with a large degree of uniformity. Absolutely uniform reactions are not to be expected from living organisms, at any rate under our imperfect control of working conditions; but when such reactions are definitely recognizable as essentially the same, the result may be judged as satisfactory. It is even more confusing to find that two or more species may react very similarly upon a particular substratum. A transfer of these organisms to a medium of markedly different composition brings out the contrasting characters, however. The desirability of recording the widest possible distinctions in such descriptive work makes necessary the use of media differing in composition as much as practicable, in such comparative cultures.

It is worthy of note that the species *P. roqueforti* and *P. camemberti*, essential to the cheese industry, have been isolated repeatedly from cheeses of widely different origin. The Roquefort species has been obtained from laboratories not concerned with Roquefort cheese studies, from ensilage, and from other substances. The same characters have been found in cultures of these two species from these variable sources under conditions in which the possibility of close genetic connection between cultures is thus remote. There seems to be little possibility of question that these species at least are well-fixed conidia-bearing forms, not cultural varieties of other species. The question at issue was not whether or how variations could be produced, but whether a particular variation is constantly produced

by a species in a particular environment. The correlative question whether the characters of a given species of mold can be permanently changed by passing through a series of cultures upon different media is involved in the same investigation. It is asserted by some workers that the physiological reactions of molds (if not also the morphology) can be changed, and that such changes persist after the return of the species to the original environment. So far as this investigation has gone such a view is certainly not supported by the conduct of the species of *Penicillium* which have been studied. In those species most thoroughly studied both the physiological and morphological reactions have appeared to be very reliable. A summary of these observations follows:

1. The same species may differ greatly in morphology and physiological reactions when grown upon different media.

2. Two species closely similar, when grown parallel in one environment, may differ characteristically when transferred to a different medium or a different set of conditions.

3. With these species the repetition of culture under particular conditions produces fairly constant morphology and reactions.

Forms arising in this way have been designated as ecads by Clements³ in a recent discussion of the "Aspects of the species question." This name is used preferably for forms whose origin is known either because produced by cultivation or under such carefully determined natural conditions as admit of full description. With species of this genus in which part at least of the morphological characters of every culture are definitely attributable to the conditions and to the chemical character of the substratum, practically all known forms would therefore be properly designated as such ecads. If described as ecads, however, each description must be limited strictly to the data obtainable upon a single medium, whereas by writing into the description the reference to media it has been found possible to include a much more complete set of characters than would readily be worked out from a single set of conditions.

These observations lead to the conclusion that the cultural description of species of molds demands the recognition of the points noted below:

1. The culture media and conditions must be described so fully as to make the repetition of the culture upon the same medium and under approximately the same conditions easily possible anywhere.

2. The habit, structure, and appearance of the colony must be given as it develops upon at least two standard media of decidedly different composition.

3. The physiological effects of the colony upon these media should be noted.

4. Full drawings or photographs should show habit as well as microscopic details of cells and cell relations.

5. Other morphological or physiological data obtainable should be given as accessory information. Very striking characters are often found under accidental or unique conditions which immediately differentiate particular species. Some of these characters come up in the ordinary course of cultural study, others are found under accidental conditions but could rarely occur in laboratory routine.

It is clear that such descriptions can only result from repeated culture of a species under constant observation. If the range of such culture has been wide, it will bring out the most striking characters and thus reduce the number of minute distinctions necessary.

NECESSITY FOR DESCRIBING CULTURE MEDIA AND TEMPERATURE CONDITIONS.

In a previous paper²⁷ the characters available from cultural studies of species of *Penicillium* were discussed. The series of observations have been greatly extended in the three years intervening. These characters may therefore be profitably reviewed, following the summary just given.

Culture medium.—The composition of the substratum is shown in this paper to affect the character of the colonies grown upon it to such a degree as to make the exact description of the medium essential. Examples of these effects may be cited to show how conspicuous these differences may be. In a medium free from certain sugars *P. duclauxi* produces upon the surface of the medium very short conidiophores with conidial fructifications, whereas when such a sugar is added numerous coremia are formed, which in well-nourished colonies often become 10 mm. in height. This species also produces a rich purple color in certain media, but not in others. Hedgcock has noted that *P. aureum* Corda (as distributed by him) produces colonies orange-red upon alkaline media, but lemon-yellow in acid media. *P. digitatum* Sacc. grows sparingly, if at all, in media offering nitrogen only as nitrates, whereas many other species grow equally well from nitrates and organic nitrogen. One species is included which produces feeble gray or brownish cultures when carbon is presented from gelatin, starch, or lactose, but becomes a clear green when cane sugar is added.

A culture medium must offer not only the proper chemical elements for fungous growth, but must offer these in assimilable form. Different species of *Penicillium* make quite different demands as to the form of food substances required, hence a description must specify the substratum accurately enough to enable the duplication of culture conditions. If, however, the proper substances in the proper form are offered, changed percentages in the concentrations of these

substances have been found to affect the growth of cultures more slowly. Most species grow normally in extremely dilute solutions, but continue to grow well or even richly until the solutions reach concentrations of considerable osmotic pressure.

Temperature.—The species studied are mostly saprophytes of wide distribution. They are therefore able to thrive within a considerable range of temperature. Comparatively few of the species tested grew normally at blood heat (37° C.). At this maximum many of them either failed to grow or were actually killed. Within the range of 12° to 30° C. the rapidity of development in most species, as indicated by the production of colored fruit, increased with the rise in temperature. The lower temperatures affect the rate of fruit formation without, as a rule, preventing such development. Cultures held at 10° to 20° C. reached a development fully equal to those held at higher temperature, only in longer periods.

HABIT, STRUCTURE, AND APPEARANCE OF COLONIES.

Many series of comparative cultures indicate that any colony of a particular species will reproduce the same characters whenever grown upon a particular medium under particular conditions. The differences between many species of *Penicillium* are so striking to the eye as to enable one familiar with them to identify the several species immediately. These same differences are, however, so difficult to define, and in many cases so transitory, as to render their expression in form to insure recognition very difficult. Recognition of species depends at best upon a series of observations of these characters throughout one, and usually more than one, generation upon two or more substrata. A discussion of these characters follows:

Fruiting period.—In comparing cultures of related forms it is found that the time necessary for development from the spore to the production of ripe conidia differs for the different species, but that periods in the different species bear a comparatively constant relation to each other when all are grown under the same conditions. Similarly the length of the growing and fruiting period varies in the several species. In some species the mycelium produces new conidiophores and masses of conidia among or overgrowing the old for a considerable length of time. In some a secondary growth of white or even colored hyphæ often overgrows the fruiting area. In others the chains of conidia once started continue to increase in length for several weeks, until the conidial fructification becomes a mass 1 mm. or even more in length. In still others but a single crop of conidia is developed and the mycelium apparently dies or is totally exhausted in a few days. In some species the center of the colony matures and dies while the margin continues to grow and produce new conidiophores for some time. The habit of the species in the production of

conidial fruit is usually a well-marked character and is often found very useful in the separation of forms found growing together.

Color.—Color and color changes are difficult to describe on account of the deficiencies in the standards of color, but they form the first character noticed. The variety of greens, blue greens, gray greens, yellow greens, and shades of brown in the genus *Penicillium* baffles one seeking descriptive terms. The shades of color peculiar to each species under oft-repeated conditions are easily recognized and are quite reliable. The alterations in the color of spores due to changing the composition of the medium, as shown in the recent work of Stoll¹⁴ and in this paper for species of this genus, and by others (Milburn,¹⁵ Bessey¹) for other genera, emphasize again the necessity of uniformity in and careful description of the culture medium. But in spite of the admitted difficulties in color description, the careful observation and record of the color of the colony at every stage of its development is very necessary to identification. This is complicated by the changes which occur at different ages of the colonies, so that it would be easily possible to place certain species in at least two of the color groups as designated by the older authors if we simply make our observations a few days apart.

The color of the mass of mycelium as observed from below ("reverse" as designated by Dierckx⁵) in cultures gives striking contrasts. This must not be confused with discoloration of the substratum which may or may not be produced by the same species in a given medium. The mycelium itself viewed in reverse has characteristic colors in certain species which are useful in diagnosis and are entirely independent of discolorations of the substratum. A colony colorless itself may color the medium brightly, while a colony bright colored itself may make no change in the color of the medium.

Surface.—As a convenient term "surface" may be used to designate the general appearance or the texture of the aerial portion of the colony. Perhaps the word "habit" would be in some measure more accurate, but that would apply also to the submerged mycelium. Comparison of the surface appearances of many cultures shows that this is one of the most stable characters when the same medium and conditions are used. For species of *Penicillium* and allied genera two types of surface will include most of the species met with.

In the one type all or a large majority of the conidiophores in the rapidly growing colony arise directly from strictly vegetative hyphae which may be submerged in the substratum or lie upon its surface or be alternately prostrate and submerged. Each conidiophore stands separately, therefore, and usually all are found to be so nearly of the same length that the surface appears to be velvety. Such a surface may be called either velvety or "strict." A strict surface may be called "closely strict" when the conidiophores are so short

that the conidial fructifications are barely raised above the surface, or it may be loose or lax if longer conidiophores give a deeper velvety appearance.

In the other type all or most of the conidiophores are lateral branches of definitely aerial hyphæ. These hyphæ and conidiophores form felted masses or loose networks of aerial mycelium for which the term "floccose" is descriptive.

Margin.—In seeking the origin of all structures direct observation of the margin of the young and growing colony is essential. Such observation determines how the fungus spreads in the substratum, the septation and measurement of hyphæ, and the origin, order of development, and relative positions of aerial structures. As the colony matures growth ceases, ripe conidial areas extend to the very margin, the masses of conidia often change color and fall apart, and the conidia-bearing branches may curl up or drop off. Some species seem to inhibit their own further growth after a short period, while in other cases they dry up the culture medium by transpiring water. Many things thus contribute to render the old colony an unintelligible mass of spores and hyphæ.

In colonies with surface strict or velvety (consisting of conidiophores only) there is a succession of structures from the center to the periphery. In the center are conidiophores with ripe conidia, marked by the colored area. This shades into a white margin of developing conidiophores and conidial fructifications, while the extreme margin consists of submerged vegetative hyphæ. The relative width of these areas and rate of the development of conidiophores and colored conidia give characteristic appearances to colonies of particular species. In some there is a broad submerged vegetative border, then a similar white band of developing conidiophores. In others the area of colored conidia extends so closely to the margin that the white border is barely discernible. In the floccose species aerial mycelium often extends as rapidly at the margin as does the submerged part. In such cases the area of coloration follows the expansion of the colony more slowly.

Although close resemblances in culture are not uncommon, the relative development of these areas is quite typical and often sharply distinctive of species. Colonies showing a broad submerged and white margin usually spread over wide areas of the substratum, whereas those bearing ripe fruit to the very edge of the growing colony rarely develop beyond restricted areas.

The gross characters already discussed have purely specific value, or may even be more closely restricted as characters distinguishing particular ecads of species. The generic characters and very important specific characters are microscopic, and include cell relations and details of spore formation.

Conidiophore.—The essential data as to conidiophores are their length, septation, the diameter of their cells, and especially their origin and relation to the substratum and to each other. Although extremes of variation in length of conidiophore may be very marked in any culture, the majority of conidiophores in any such culture approximate an average length. This length to be most reliable must be taken from the origin in another hypha to the lowest branch of the fructification. If the conidial fructification were counted into the length, the length of conidiophore would in many cases be doubled with the maturing of the spores. The actual length, however, is little changed with such maturity. Valid data on these points can be secured in many species only by direct observation of the undisturbed colony in the air under the microscope, instead of by the study of fluid mounts. This is equivalent to saying that Petri dishes, or other vessels which can be uncovered for study, must be used. The student must expect, therefore, to make many cultures and jeopardize the purity of one such culture every time he undertakes its proper examination.

Conidial fructification.—For lack of a better term conidial fructification may be defined as including the chains of conidia, the conidiiferous cells, and the branches bearing them back to their junction with the conidiophore proper. In a species of *Penicillium* fructifications vary greatly in detail, so that satisfactory illustration becomes difficult. Comparison of large numbers of fruiting branches in many cultures of the several species establishes specific types of appearance which can be shown in a conventionalized series of sketches for each species. The data found of value have been the mode of branching, the measurements of branches and conidiiferous cells, the relation of these to each other, the arrangement of the chains of conidia with reference to each other, and the measurement and appearance of the fructification as a whole. These penicillate fructifications vary from close columns of spores arising from single verticils of cells borne directly upon the apices of the conidiophores to widely spreading "brooms" whose numerous divergent chains arise from verticillately or complexly arranged branches from the original conidiophores. In the study of these conidial fructifications mounting in fluid commonly greatly disarranges these complex masses of branches and spore chains. Direct observation of the undisturbed colony is therefore essential to a correct conception of the habits of the species, though the details of branching and spore bearing must be sought in fluid mounts.

The term "conidiiferous cell" is used for the cell at the base of every chain of conidia in preference to "sterigma," as often used, or to "basidium" as used by Stoll.²⁴ The term was proposed by Professor Atkinson as meeting the objection that basidium implies a relationship not justified in fact, while sterigma usually designates not a spore-

producing cell but a spore-bearing process of a cell, hence is properly applicable to the narrowed apices of these very cells. In the Latin descriptions the term "basidium" has been retained, however.

Conidia.—The usual data with reference to the conidia are of equal service in our cultural studies, viz, shape, size, color, arrangement, markings, mode of germination, and conditions of growth. A record of the changes in color at different ages of the colony is essential. Completeness in observation is as necessary in spore characters as elsewhere. The variations in the size of conidia are notable in some species; in others conidia appear to be either globose or elliptical, even in the same chain. Where the outer cell wall is marked or spiny these markings often do not appear until the conidia are fully mature.

PHYSIOLOGICAL EFFECTS UPON MEDIA.

Certain physiological effects of fungus growth which are incident to the use of ordinary culture media have been found to be reliably characteristic of species. Some of these reactions are so conspicuous as to aid greatly in separating nearly related organisms. Such reactions as have been found uniform and definite or unique are introduced into the technical diagnoses of species in this paper. Other physiological data are appended as accessory cultural information.

Among the data observed in repeated series of comparative culture are the following: Odor, litmus reaction of medium at different stages of growth, liquefaction of gelatin media, the production of coloring substances in the media, the changes produced in milk, and the production of drops of transpired fluid upon surface of colony. Among the accessory data, observations upon carbon assimilation, upon proteolytic reactions, and upon the production of enzymes have been made for certain species.

Odor.—The production of definite odors by colonies is confined to a small number of species, and even among these often to particular media. When definitely present it is a character immediately recognizable and in certain species diagnostic; in others it associates the organism at once with a particular group of species.

Litmus.—The use of an indicator in the medium gives in very many species a sharp reaction. The value of this reaction is more narrowly restricted, however, than is indicated in previous papers (Thom^{25, 26, 27}). The introduction of sterilized litmus or azolitmin into complex media brings contradictory results when the composition of the medium is slightly altered. If, for example, a solution of pure gelatin in distilled water be used as a nutrient, the reactions are definitely alkaline, with very few exceptions, which are just as definitely acid. The gelatin solution itself is acid. The alkaline reaction indicates that the products of the digestion of gelatin in such cases are of alkaline nature. If, however, the gelatin solution be neutralized and

5 per cent cane sugar be added, an equally large majority of the same species cause an acid reaction. Some few remain alkaline. These species growing upon cane sugar produce acids in quantity both to neutralize the alkaline products of the decomposition of gelatin and to change the reaction of the mass. The litmus reaction has been found generally reliable in a medium composed of 15 per cent gelatin in distilled water, in lactose-peptone gelatin after Conn's⁴ formula, and in potato agar during four years of cultural work where many successive lots of media have involved the use of materials from different sources. In complex media the decomposition products of organic nitrogenous constituents and those of carbohydrates tend either to neutralize each other or to intensify the reactions, but add greatly to the difficulties of analyzing the results. The litmus reaction therefore may be used with the simplest organic media, but is best applicable to cultures in synthetic media where analysis of the results is feasible.

Gelatin.—The liquefaction of gelatin media by an organism in culture shows its ability to produce a particular form of proteolysis. This reaction is so conspicuous and so adaptable to cultural use that it has been recorded in all cultural studies. Various investigators have pointed out the limitations of this reaction. In cultivation nearly all of the species of *Penicillium* have been found to grow well upon a 15 per cent solution of gelatin alone in distilled water. Comparison of the results in this medium with liquefaction of several forms of gelatin media experimented with showed essential agreement. Since the advantages all lie in the simplification of formulæ, the data as to liquefaction by these species have been compiled from series of parallel cultures of all the species upon 15 per cent gelatin in distilled water.

The value of this reaction is measurably vitiated by the fact that any species which can subsist upon gelatin alone must be capable of more or less proteolytic action upon it. The results of observation confirm this statement. The value of these observations therefore depends upon the indication of the comparative rate of activity of different species in inducing proteolysis, not upon the presence or entire absence of this action. Numerous tests, involving a range from 15° to 25° C., show that slight changes of temperature affect this reaction only to the extent to which they affect the rate of growth, but do not disturb the comparative value of the data obtained. Within this range of temperature the most active species produce liquefaction in 5 to 8 days, other vigorous liquefiers require 8 to 15 days, while many species producing no liquid or but traces of softening in 15 days produce a gradual liquefaction in the succeeding 2 to 4 weeks. In studying species of *Penicillium*, liquefaction of gelatin has been found to separate such species as produce this proteolysis within

the time needed for other observations upon these cultures—about 15 days. Where liquefaction occurs during the active growth of the colony it definitely indicates the secretion of ectoenzymes capable of this digestion. Long-deferred liquefaction may result from such secretion or from the liberation of endoenzymes by the disorganization of mycelium.

Color production.—Certain species cause marked changes in color in some of the substrata in which they grow. Such reactions are usually produced upon certain media and not on others. They are therefore selective reactions. For example, one species produces a bright yellow color in media containing milk sugar, such as milk or peptone-milk sugar-gelatin; but no color in plain potato agar. Some of these colors are soluble in alcohol to form brightly colored solutions. The chemical nature of these substances has not been studied, but the reactions themselves have been thoroughly tested for a few species. In these species color production uniformly follows the proper culture conditions.

Production of fluid upon surface of colony.—The presence upon the surface of the colony of large drops of transpiration water which may be colorless or brightly colored by excreted products is common. Although a transient character dependent to some measure upon the humidity of the air in the culture vessel for its prominence, there is much difference between species in this particular.

Technical descriptions of these fungi have always been based, theoretically, upon morphology only. In such descriptions the host or substratum has been more or less definitely indicated. Where the organisms are parasites or saprophytes closely restricted to particular substrata and conditions, this practice can perhaps be justly supported. With omnivorous cosmopolitan saprophytes cultural study quickly shows morphology to be affected greatly by changed conditions or altered composition of media. Further, very many of these organisms have no known special habitat. A description based upon a specimen of one of these species found in the field, without comparative culture, might possibly contain some peculiar character which would identify a specimen of the same species when next found, but this is only a possibility. In practice the descriptions we have are nearly useless.

The introduction of cultural study carries with it the necessity of recognizing at least the most striking physiological data. With these species of *Penicillium* such data have been found as reliable as morphology. Further, the careful definition of morphology and reactions upon specified substrata under known conditions is only amplifying and rendering definite the two items—habitat and locality—which have always been included in plant descriptions. In recent years the students of ecology and of experimental evolution

have shown these to be much more important in flowering plants than was formerly supposed. The introduction of these characters is thus in full conformity with the best systematic practices of to-day, where the life history of the species is investigated as fully as possible.

CULTURE MEDIA.

In developing the descriptions presented the following media have been used:

1. Potato agar. This was made as described in another paper²⁵ (p. 7). Essentially this is simply a potato infusion to which agar-agar is added in any proportion desired. Since this medium is free from sugar and very dilute in nutrient value, sugar may be added to cultivate certain species.

2. Bean agar. The directions for making bean decoction were obtained from Mazé at the Pasteur Institute in Paris. Common white beans are heated in five volumes of water. Boiling is stopped just before the swelling of the cotyledons would rupture the seed coats. This gives a clear, slightly yellowish liquid which filters readily yet contains sufficient nutrients to grow many species normally. Agar may be added as desired. Since this decoction is poor in available carbon, the addition of sugar is often desirable for many species.

3. Peptone-milk sugar-gelatin, as described by Conn.⁴ This standard bacteriological formula, with and without litmus, was used in developing the original draft of these descriptions.

4. Fifteen per cent gelatin in distilled water. As a stock medium, this was used without neutralization. Comparative cultures neutralized with NaOH seemed less adapted for most species than unchanged gelatin. With few exceptions the common species of *Penicillium* grow readily in plain gelatin and give the same reaction as in Conn's more complex formula.

5. Synthetic media. A. W. Dox, chemist to this investigation, has modified Czapek's formula for a synthetic medium, intended to present in a nearly neutral solution unaffected by sterilization the elements necessary for fungous growth. In stock form neither nitrogen nor carbon is presented in this fluid. It has consequently been found an excellent means of testing the availability of these elements in various forms. For convenience this will be referred to as Dox's solution, the formula of which is—

Distilled water.....	centimeters..	3,000.0
Magnesium sulphate.....	grams..	1.5
Dipotassium phosphate (K_2HPO_4).....	do....	3.0
Potassium chlorid.....	do....	1.5
Ferrous sulphate.....	do....	.03

In the work here reported nitrogen was added as sodium nitrate, 6 grams. Parallel experiments in which monopotassium phosphate (KH_2PO_4), which gives a perfectly clear solution of a strongly acid reaction, was substituted for dipotassium phosphate, K_2HPO_4 , gave no advantages in culture to offset the advantages of a neutral medium except the disappearance of the traces of precipitated magnesium phosphate. The availability of carbon in any organic form can be tested readily in this solution. Solidified media are obtained by the addition of agar.

NOMENCLATURE.

THE GENERIC NAME.

The generic name, *Penicillium* Link, is held in this paper in its hyphomycete sense to designate all species which continue to propagate themselves for an indefinite number of generations by penicillate asexual fructifications. Such grouping does not imply the author's belief in the phylogenetic relationship of all such forms. The penicillate type of fructification is a definite character which binds together in this way into a "form-genus" a large number of cosmopolitan and omnivorous saprophytes, very few of which are known to produce sexual fructifications. Within this heterogeneous group, several series of forms possessing particular groups of characters have been separated and generic names have been based by some workers upon such segregation. Wehmer²⁸ founded the genus *Citromyces* upon two such species causing citric acid fermentation in sugar media, with its morphological basis in the presence of a single whorl of conidia-bearing cells at the apex of the conidiophore. Further study shows that the presence of this character alone would group together forms not so closely related to each other as to other species of *Penicillium* lacking this character. It seems best, therefore, to use the name *Penicillium* to designate the entire group and leave further investigation to establish permanent genera when really genetic relationships shall have been discovered.

NOMENCLATURE OF SPECIES.

In considering the actual problem of nomenclature of species several positions may be taken. It is not difficult to find published descriptions of single species in this genus which are sufficiently indefinite to include a large percentage of all the known species. As a rule, the morphological characters of the various forms under cultivation would not exclude them from half a dozen of the older species so far as current descriptions go. In many cases there would not, however, be the least reason for the adoption of one name in preference to another. Shall the investigator adopt for his material

a name previously used when he has little or no reason to believe that he has the organism originally described under that name? Granting the apparent impossibility of contradiction, he might find it a safe practice. If, however, contradiction should arise, the position becomes entirely indefensible. In case there is a fair probability that the forms are identical, the use of the old name may be justified perhaps without direct proofs. The alternative position calls for the complete description of the form studied and its distribution in culture to different centers of cultural work under a new specific name associated with this definite material and description. It can not be contended that these organisms are new to science, for it is entirely possible that certain of them have formed the basis for already published descriptions. This course can be justified by the contention that such publication applies to definite material, available for examination, culture, and comparison by others, and that there is less probability of confusion from such publication than would ensue from the use of names long published, without any real evidence of the identity of the organisms with those originally studied. After careful examination of all material available and consultation with many workers in closely similar fields, new names are attached to such forms as by continued cultural study appear to be sharply marked species but not identifiable by older descriptions.

The name *P. glaucum* is not used. Careful examination of literature and of cultural material from several sources, together with conferences and correspondence with investigators in numerous laboratories, does not afford evidence as to what form was originally used by Link or even secondarily by Brefeld² under this name. The name as used at present seems to be applied collectively to the common green forms which under examination are quickly found to be not one but several species. Further study may give some indication as to where the name really belongs, but until that time there is little profit in applying it to any particular form. It might, perhaps, be withheld until some worker succeeds in repeating Brefeld's classic studies in ascus production, and then applied to the form so found.

The present paper is not intended to be a monograph of the genus. It is presented as a report covering several thousands of cultures of a group of common forms, in the hope that the descriptions and key offered may be useful to others. The author has included studies of as many authenticated cultures of the more recently described forms as it was possible to secure. The verified cultures secured have already been listed. Much assistance and advice were freely given by Dr. C. Wehmer. On the other hand, it was impossible to secure cultures of the species recently described by Oudemans,¹⁷ or those listed by Diereckx;⁵ and one recently described by Peck¹⁸ also appears to be lost. No claim to completeness can be

made, though the material may possibly form the nucleus of a real monograph later.

In the examination of the early literature of this genus the author is greatly indebted to Dr. C. L. Shear, of the Bureau of Plant Industry of this Department, for the use of books and for much careful cooperation in examining and discussing the subject of types and descriptions.

THE TYPE SPECIES.

Link¹¹ in his "Observationes," published in 1809, established the genus *Penicillium* to include the common green molds having a penicillate type of conidial fructification—a conidiophore branching more or less complexly at its apex, such branches becoming or being tipped by cells, each of which produces a chain of conidia. The whole produces a brush-like appearance, the chains of conidia serving as the hairs or bristles of the brush. This generic name has been universally accepted to include in a form genus all species reproducing themselves indefinitely by such conidial fructifications. However doubtful we may be as to the forms originally examined, there is no question that we know the general type of structure which Link intended in his description of the genus.

Under the genus *Penicillium*, Link placed three species. The first species listed was *P. glaucum*, but the description given is equally applicable to many different forms. This was noted as frequent in decaying bodies and said to be most closely related to *P. expansum* (the third species listed), of which he suggests his material may have been but undeveloped specimens.

The second species, *P. candidum*, is described as producing round colonies, with mycelium and spores white, upon decaying fungi and herbs. Although many authors have used this name for material from different sources, and Morini¹⁶ has described an ascigerous form under this name, there has been no means of determining what form Link had in mind when writing his description. Many forms will produce white mycelium and spores under special conditions, while but one of those examined has been shown to do this under all conditions, and this one is so specialized in its habit as to be excluded by Link's statement of habitat from the application of this name. Numerous authors have suggested that the *P. "candidum"* forms are probably colonies of species, colorless under special conditions, but green under other cultural conditions.

The third species, *P. expansum*, is slightly better described, while its habitat is primarily given as rotten fruit, although the author has manifestly extended his use of the name to forms growing upon other substrata which he believed to be identical with the fungi grown upon decaying fruit.

On page 19 of his "Observationes" Link describes the genus *Coremium* with a single species, *C. glaucum*, which he specifies as found upon decaying fruits. This fungus can be traced through a connected series of publications giving descriptions and figures which show the original conception to include (if not entirely to be drawn from) the large green coremia which develop upon apples and related fruits decaying in storage.

This organism is figured by Greville⁶ as *Floccaria glauca*; it is cited by Fries⁷ as a variety of *Penicillium crustaceum*, from which he has "seen it originate upon apples in the autumn." It is twice referred to by Corda in *Icones Fungorum* (Vol. II, p. 17); he cites *Coremium glaucum*, *C. citrinum*, and *C. candidum* as synonyms; again in *Prachtflora* (p. 54, taf. XXV, figs. 3, 4, 17, 18, 19, 20, and 21) under the name of *Coremium vulgare* he manifestly had this same species, although he groups it with figures which appear to be different organisms.

These figures and descriptions cited are definite enough to show that workers contemporary with Link applied the name *Coremium glaucum* Link to the coremiform rot of the apple. As a result of observations of living material Fries considered this only a form of *Penicillium crustaceum*, which he made to include *P. glaucum* and *P. expansum* Link.

The present writer has collected this fungus upon decaying apples and related fruits repeatedly in America; also upon pears and mespilus in Hanover, Germany.

Repeated cultures have shown that the ability to produce coremia is a definite character of this species, recognizable under many conditions of culture, but not shown under other conditions. The same culture will commonly show both simple penicillate fructifications and coremium production. The species must therefore be regarded as one of the several species of *Penicillium* which always produce coremia under proper cultural conditions.

Examining Link's species of *Penicillium*, we find that he specifies *P. expansum* as primarily found upon rotten fruit. *P. expansum* Link clearly included *Coremium glaucum* Link, therefore, probably with others; but from its known abundance in Germany there can be little question as to this organism forming in part, at least, Link's original conception of this species. Later (1824) in *Species Plantarum*, Tomus VI, page 70, Link¹¹ redescribes *P. glaucum* and includes in it the *P. expansum* of his *Observationes*. In this discussion Link broadens his description of *P. glaucum* to include all green forms found in decaying substances, upon the assumption that all such forms are but a single species. It is evident that the earlier description *P. expansum* Link included this species with sufficient restriction

to justify reviving the name *P. expansum* Link and limiting it to the penicillium rot of apples alone.

P. expansum Link (in part), the penicillium rot of apples, would therefore stand as the type species of the genus.

The technical rules for the establishment of type species are in this way satisfied. The kind of plant used by Link is perfectly well known. To say just which forms he had in hand may be impossible, but the form we are discussing was certainly one of them.

EXPLANATION OF DRAWINGS.

The drawings of the species described have been made with the Bausch & Lomb camera lucida in all cases except such as are marked diagrammatic or partly diagrammatic.

For those with the magnification of 140, the lenses used were the Bausch & Lomb 1-inch ocular and the two-thirds objective; for those of 900 the lenses were the Bausch & Lomb 1-inch ocular and the one-eighth objective; for those of 1,400 the lenses were the Bausch & Lomb 1-inch ocular and the one-twelfth objective; for those of 1,600 the Spencer No. 12 compensating ocular and the Zeiss 3 mm. apert. 1.30 apochromatic. This apochromatic was also used in a few cases at a magnification of 900; these are indicated in the legends. All figures at magnification of 140 were drawn from the exposed surface of the undisturbed colony in Petri-dish cultures. All other figures were made from fluid mounts or hanging-drop cultures (for spore germinations).

The series of sketches at 140 magnification are made with uniform methods, so that comparison of species in culture can be much easier than from figures made at different magnifications.

PENICILLIUM EXPANSUM Link.

P. expansum Link (in part), emended Thom=penicillium rot of apples and allied fruits.

Syn. *Coremium glaucum* Link, Observations, p. 19; Icones, V, fig. 31, 1809.

Floccaria glauca Greville, Scottish Flora, pl. 301, figs. 1-4.

Penicillium glaucum Link (in part), Species Plantarum, VI (1824), p. 70.

Coremium vulgare Corda (in part), Prachtflora, p. 54, Pl. XXV, especially figs. 3, 4, 17, 18, 19, 20, and 21.

Possibly *P. elongatum* Dierckx.

Colonies upon gelatin and potato or bean agar, green becoming gray-green and slowly brown in several weeks (especially when exposed to light), floccose, with concentric zones tufted with short, loose, coremium-like aggregations of conidiophores, not over 1-2 mm. in height except in old cultures containing sugar, broadly spreading with broad white margin in growing colonies. Reverse somewhat brown. Conidiophores either very short lateral branches of aerial hyphæ or very long (1 mm. or more), arising singly or grouped with others to form coremia. Conidial fructifications consist of 1 to 3 main branches bearing verticils of branchlets supporting crowded whorls of conidiiferous cells, 130-200 by 50-60 μ at base in cultures without sugar, with sugar

continuing for some weeks to produce great numbers of conidia which come to form masses perhaps 1 mm. in thickness. Conidiiferous cells 8-10 by $2-3\mu$. Conidia elliptical to globose 2 by 3.3μ or $3-3.4\mu$; green, homogeneous, persisting in chains when mounted. Colonies begin to liquefy gelatin slowly after about 10 days and continue until it is completely liquefied. Grows readily and rapidly upon all common media.

Occurs characteristically upon decaying apples and other pomaceous fruits, where old colonies often produce coremia 1 cm. or more in length and very large.

Collected at Ithaca and Geneva (Eustace), N. Y., at Middletown and Storrs, Conn., upon apples; upon pears and Mespilus at Hanover, Germany. Often appears as a contamination in fungous cultures.

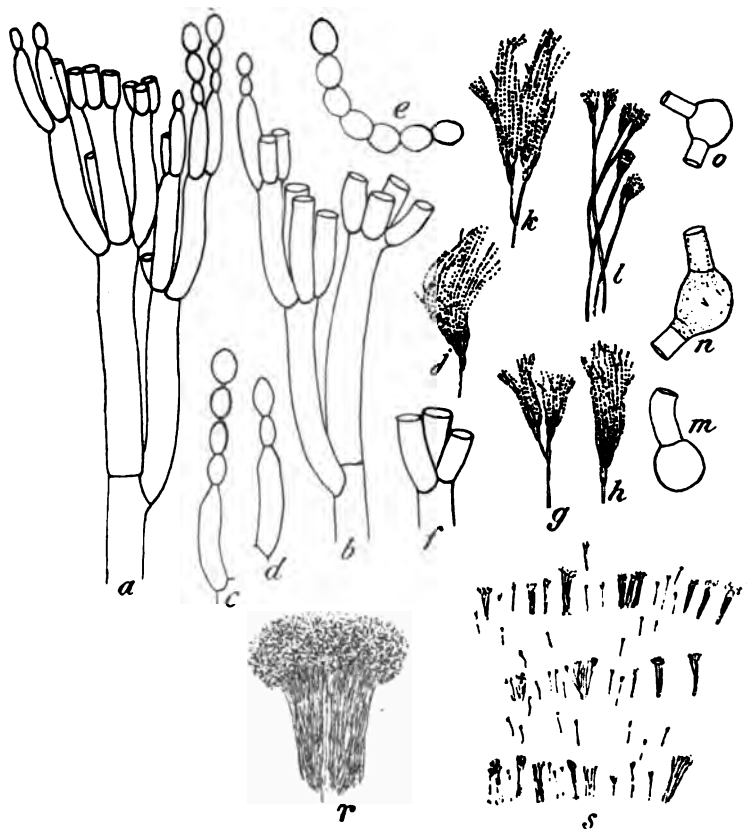


FIG. 1.—*Penicillium expansum* Link: *a*, *b*, *f*, branching and arrangement of branches of conidial fructification ($\times 900$); *c*, *d*, *e*, conidiiferous cells and conidial chains ($\times 900$); *g*, *h*, *j*, *k*, *l*, sketches of fructification ($\times 140$); *m*, *n*, *o*, germination of conidia ($\times 900$); *r*, *s*, sketches from photomicrographs, showing in *s* loose aggregations of conidiophores beginning to develop in zones which become coremia like *r* (coremium *r* was 1 mm. in height; $\times 35$).

Many references in the literature to *P. glaucum* Link and *P. crustaceum* (L.) Fries refer to this species. Unless, however, such citations directly refer to the presence of coremia, or to the association of the organism with the decay of pomaceous fruits, or both, there is no means of fixing the application of such names to this species. Since Link¹¹ in 1824 lumped into his species *P. glaucum* every kind of green

penicillium for which he could find references, the myth of "the common green mold" seems to have had pretty general acceptance, although we find here and there a protest against this view.

A culture of this species can always be obtained from apples decaying in storage, upon which usually well-developed coremia can be found if proper search is made. The wide distribution of the organism as noted above and as seen in the literature justifies belief in its general distribution. Once carefully observed in cultures, the investigator will usually recognize the organism on sight when it appears in his cultures even as a contamination of other species. The odor of this species is so distinctive as to assist greatly in identifying it in accidental cultures. It does not produce the yellow color in the substratum as described by Lindau¹⁰ for *P. glaucum*, nor are its spores globose from the first as Wehmer²⁰ records them for that species. It seems so very well characterized as to justify sharply separating it from the other green forms.

CULTURAL DATA.

Color, green or gray-green; color of reverse, yellowish to somewhat brown; color in media, colorless or yellowish brown (milk).

Odor, "fruity" in all or nearly all cultures.

Fifteen per cent gelatin in water, good growth, with loose and ill-defined but abundant coremia; liquefaction slow, more or less liquid in 2 weeks; litmus reaction alkaline or neutral. Potato agar and bean agar, characteristic colonies, gray-green with more or less coremiform bundles of conidiophores. Potato plugs, characteristic colony.

Raulin's fluid agar, characteristic colony with concentric rings of broad coremia. Raulin's fluid, characteristic colony. Cohn's solution, slight growth, few coremia, brownish below.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, good coloring up to 50 per cent sugar, acid reaction, conidiophores in dense areas with few definite coremia. Lactose 3 per cent, slow development, about half normal. Levulose 3 per cent, typical, alkaline, or neutral reaction. Galactose 3 per cent, typical, acid reaction. Glycerin 3 per cent, slow development, increased greatly by adding sugar. Potato starch 3 per cent, fair growth, no coremia. Butterfat, rich typical growth.

Milk, typical colonies, coremia in a ring at glass; curdling (0.25 per cent calcium chlorid added) in 1 week; digestion rather slow; color in milk yellowish brown.

At 37° C., no growth, culture grew when cooled; at 20° C., good growth.

The coremia of this species are especially characteristic of old cultures in which drying has begun. In fluid cultures they are commonly attached to the glass above or at the very top of the fluid.

PENICILLIUM ITALICUM Wehmer.

Beitr. z. Kennt. einh. Pilze, Jena, 1895, p. 68, t. II.

Colonies on plain gelatin and potato or bean agar bluish green, becoming gray-green when old (bright bluish green on cane-sugar media and citrus fruits), broadly spreading, aerial portion composed at the broad margin almost entirely of conidiophores, but becoming slightly floccose in the center. Reverse of colonies dark brownish, often almost black in media containing sugar. Conidiophores from short (100 μ) to very long (600 μ), averaging perhaps 250 μ , arising either directly from substratum or as branches of aerial hyphae. Conidial fructifications up to 300 μ or more in length, consisting usually

of a main branch and one lateral branch, each producing a whorl of branchlets bearing crowded verticils of conidiiferous cells, 12–14 by 3μ . Conidia breaking off in masses in handling old cultures, which rise in clouds when shaken. Pronounced odor in cultures containing cane sugar. Chains of conidia loosely divergent, long; conidia 2–3 by $3\text{--}5\mu$, cylindrical to elliptical or slightly ovate, clear green by transmitted light, very variable in size but usually within the limits given. Masses of spores continue to increase from 2 to 3 weeks. Petri-dish colonies partially and slowly liquefy gelatin (12 to 20 days). Numerous white sclerotia are produced upon the surface of

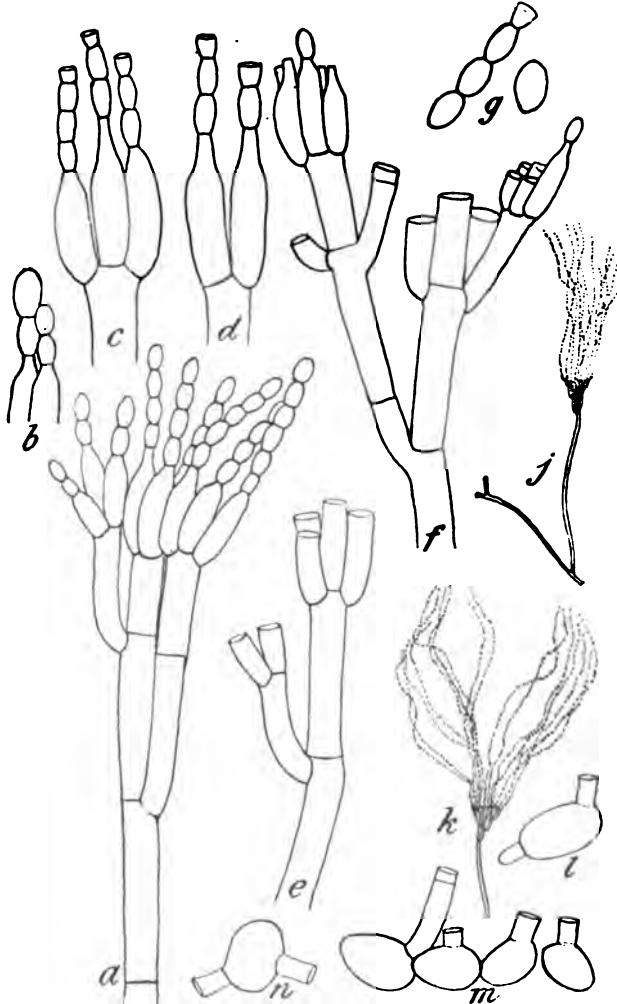


FIG. 2.—*Penicillium italicum* Wehmer: a, b, c, d, e, f, g, types of branching, formation of verticils of conidiiferous cells and conidial chains (a, e, f, $\times 900$; b, c, d, g, $\times 1,400$); j, k, sketches of conidial fructifications ($\times 140$); l, m, n, swelling and germination of conidia ($\times 900$).

the medium after 2 to 3 weeks' growth, especially upon fruits and other acid media rich in sugar.

Cosmopolitan; characteristic of decaying oranges, which become bright blue-green with this mold as contrasted with the olive-green species which is often associated with it upon the same fruit. These contrasting colors are illustrated by Wehmer.³¹

In his discussion of this fungus Wehmer³¹ shows that the blue-green rot of citrus fruits is a different species from the similarly colored apple rot. His description seems to be the first recognition of this species as different from the green molds occurring constantly upon all kinds of food. This species has been discussed as *P. glaucum* in recent papers (R. E. Smith,³² Powell³⁰), where its agency in the decay of citrus fruits is very fully considered.

Cultures were obtained by the writer from oranges in Hanover, Germany, and identified by the describer, Dr. C. Wehmer. It has since been repeatedly observed and collected in America. Pure cultures can always be secured by finding decaying oranges in the market which have the blue-green areas of rot just beginning to appear upon them. These areas are usually blue-green in center surrounded by white areas which are usually grouped into little white patches toward the vegetative margin and the whole superficial colony surrounded by an area of soft, watery rot. Very often such colonies when older become much contaminated with the olive-colored rot, given in this paper as *P. digitatum*.

CULTURAL DATA.

Color, clear bluish-green on sugar media, shades of gray-green without sugar; reverse of colony commonly brownish in areas; color in media none or slight.

Odor distinct upon media containing cane sugar, none on lactose or media free from sugar.

Fifteen per cent gelatin in water, medium growth; liquefaction, none until several weeks old, then partial in acidified cultures; litmus reaction fairly alkaline. Potato agar with lactose, rather thin gray-green colonies, not vigorous, acid reaction. Potato plugs, pale but characteristic. Raulin's fluid, typical colonies. Cohn's solution, germination only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, good colonies up to 50 per cent with alkaline reaction. Lactose 3 per cent, slow growth but typical, with acid reaction. Lactic acid 0.9 per cent, small growth. Levulose 3 per cent, not normal. Galactose 3 per cent, medium growth, acid reaction. Glycerin 3 per cent, no growth; growth began when sugar was added. Butterfat, growth slow.

Milk, good growth; curdling (0.25 per cent calcium chlorid added), ten days; digestion partial and slow; color slowly brownish or yellow-brown.

At 37° C., killed; at 20° C., good growth.

PENICILLIUM DIGITATUM Saccardo.

The olive-green orange rot, *P. digitatum* Sacc., in Mycotheca Italica, No. 986, Herbarium U. S. Department of Agriculture; in Sylloge Fungorum, Vol. IV, p. 79; in Fungi Italici, No. 894.

= *P. olivaceum* Wehmer, Beitr. z. Kennt. einheim. Pilze, pp. 73, t. II, Jena, 1895.

? *Mucor cæspitosus* L., in Species Plantarum (1753), II, p. 1186, based upon Micheli, tab. 91, fig. 3.

? *Monilia digitata* Fries, Systema Mycologicum, III, p. 411.

Colonies on sugar gelatin and potato or bean agar grayish olive, irregularly shaped from the unequal growth and branching of rather few hyphæ, aerial portion consisting only of very short conidiophores and conidia. Reverse of colony commonly shows brown to black colors. Conidiophores rising directly from the substratum, 30-100 by

4–5 μ , usually very short. Conidial fructification a few tangled conidial chains up to 160 μ in length, borne upon conidiiferous cells 13–16 by 3–4 μ . Conidia cylindrical to almost globose, 4–7 by 6–8 μ (at times 6 by 10 μ), often uneven in size and shape in the same chain. Colonies do not liquefy sugar gelatin except at times partially in cultures three weeks old or more. Litmus reaction acid. Grows readily on organic media, but shows a very pronounced affinity for such media with high percentages of sugar, in which it produces a strong odor. Refused to grow in synthetic media containing nitrogen as sodium nitrate.

Cosmopolitan upon citrus fruits, distinguished from *P. italicum* by the sharp contrast of its olive color with the blue of the other. Collected in Hanover and verified by Dr. C. Wehmer. Received from Prof. P. H. Rolfs in Florida. Seen upon decaying oranges everywhere. Pure cultures can always be secured from the common market fruits.

Nomenclature.—Wehmer³¹ (1895) gives the first adequate discussion of the decay of citrus fruits by the agency of species of *Penicillium* in which the forms found were shown to be distinct species associated constantly with these fruits instead of common green species accidentally occurring upon these fruits. For the olive-green form the name proposed, *P. olivaceum*, is descriptive, but had already been used by Corda (Icones, III, p. 12, t. II, fig. 35) for a species afterwards transferred to *Hormodendrum*. The name *P. olivaceum* Wehmer is therefore not tenable by present rules of nomenclature.

P. digitatum is the name used by Saccardo, as is shown by the specimens distributed by him (Mycotheca Italica, No. 986) previous to Wehmer's work. In his description and synonymy he cites the name from Fries (Systema Mycologicum, p. 411), where it appears as *Monilia digitata*, transferred by Saccardo to *Penicillium*. But Fries cites his use of the name from Persoon¹⁹ (Synopsis Fungorum, p. 693), who bases his description of *Monilia digitata* upon Micheli's¹⁴ figure and description (Nova Plantarum Genera, p. 213, pl. 91, fig. 3). Here, under the name of *Aspergillus*, Micheli figures and describes as No. 8 a fungus which may be taken for a *Penicillium*, which is said to have been found upon semi-putrid lemons. This figure is cited by Linnæus in Species Plantarum (1753), II, p. 1656, as the basis of his *Mucor cæspitosus*.

If we accept Saccardo's citations as correctly giving the origin of the name and tracing it back to Micheli, we must abandon the name he uses and adopt the name given by Linnæus—*Mucor cæspitosus* = *P. cæspitosum* (L.). Careful scrutiny of Micheli's figure gives no possible means of identification. Aside from identification from preserved specimens which are not cited by the authors in any case, there is little possibility of showing what any of the authors commonly cited, for this species may have had until we come to the description given by Saccardo in the Sylloge (IV, p. 79), and the material distributed by him in Mycotheca Italica, which I have seen and which is certainly this fungus. I have therefore continued the use of Saccardo's name in this case because he has given the first

description based upon tangible material, but have rejected his citations. The following are the principal citations which have been given as referring to this species:

Micheli, *Genera Plantarum* (1729), pl. 91, fig. 3.

Linnaeus, *Species Plantarum* (1753), II, p. 1656.

Persoon, *Observations*, p. 41.

Persoon, *Synopsis Fungorum*, p. 693, *Monilia digitata*.

Fries, *Systema Mycologicum* (1829), III, p. 411, *Monilia digitata*.

Saccardo, *Fungi Italici* (1881), No. 894, and *Sylloge*, IV, p. 79, *P. digitatum*.

Wehmer, *beitr. z. Kennt. einheim. Pilze*, p. 73, taf. II.

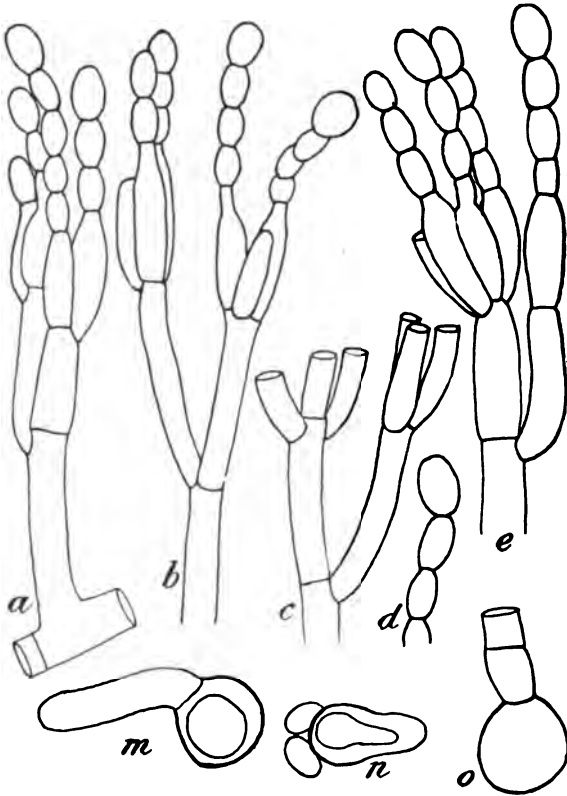


FIG. 3.—*Penicillium digitatum* Saccardo: a, whole conidiophore and fructification; b, c, d, e, types of branching and formation of conidia; m, n, o, germination of conidia; all $\times 900$.

The only reason for retaining the name *P. digitatum* and ascribing its authorship to Saccardo is that the description cited is the first one which we can ascribe definitely to material of this species. If, as said above, his citation of literature proves to be correct, the name he gives becomes untenable by present rules of nomenclature, and must be changed to *P. cæspitosum* as given by Linnaeus, although there is no evidence that Linnaeus actually examined material of this species. Since there is more or less doubt about usages previous to Saccardo, and since the name given by Saccardo has become asso-

ciated with studies of this species in recent literature (Powell,²⁰ Smith²¹), this name is allowed to stand, at least until we have more information.

CULTURAL DATA.

Color, shades of olive green; reverse brown or dark brown, especially in sugar media; color in media, none or slightly yellowish.

Odor, associated with smell of rotting oranges, strongest upon cane-sugar media.

Fifteen per cent gelatin in water, weak growth, not adapted for this species; liquefaction none, slowly accomplished in gelatin to which sugar is added; litmus reaction acid. Potato agar and bean agar, colonies as described above, rather poor growth which becomes enormously increased upon the addition of cane sugar. Potato plugs, excellent growth. Raulin's fluid, colony colorless. Cohn's solution, germination only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, germinated only. Lactose 3 per cent, germinated but no growth. Lactic acid, 0.9 per cent, germinated only. Levulose 3 per cent, germination only. Galactose 3 per cent, slight growth. Glycerin 3 per cent, germination. Potato starch 3 per cent, slight or no growth. Butterfat, germination only. Conidia inoculated into this fluid with various sources of carbon germinated but never developed normal colonies.

Milk.—This species never produced vigorous growth upon milk.

At 37° C., killed; check at 20° C. grew well.

PENICILLIUM ROQUEFORTI Thom.

U. S. Department of Agriculture, Bureau of Animal Industry, Bul. 82, pp. 35-36, fig. 2, 1906.

Report Storrs Agricultural Experiment Station, 1905, p. 111.

Syn. *P. glaucum*, various authors—not Link or Brefeld.

Colonies on potato agar or lactose gelatin quickly turning green, becoming a dirty brown when old, velvety strict, indeterminately spreading by large main radiating, branching hyphæ, giving a somewhat uneven or indefinite margin, which gets a white, fibrous, almost spider-web appearance from its alternation of submerged parts of hyphæ with short prostrate aerial loops. Reverse of colony yellowish white. Conidiophores arising separately and in acropetal succession from the growing parts of submerged hyphæ (comparatively few aerial parts, but some), 200-300 μ septate. Fructification 90-120 μ or at times 160 by 30-60 μ at broadest place, usually appearing double by the divergence of the lowest branch; branchlets ("basidiophores") irregularly verticillate, bearing crowded verticils of appressed conidiiferous cells (basidia), 9-11 by 2.5 μ with long, divergent chains of conidia. Conidia bluish green, cylindrical to globose, smooth, rather firm walled, 4-5 μ in diameter, germinating by a straight tube. Colonies do not liquefy sugar-gelatin, though they soften it somewhat. Fungus on plain gelatin or potato agar changes litmus from red to blue very rapidly and strongly almost from the beginning of the growth. Fruiting period short, but one crop of spores upon the mycelium. Cosmopolitan and omnivorous, or nearly so. Characteristic of Roquefort and related types of cheese.

The mold of Roquefort and related types of cheese has been commonly designated in dairy literature as *P. glaucum* Link. Such citations refer to this fungus, though many times referring to it as "the common green mold." Although it is not restricted in its habitat to cheese, this species is so identified with the ripening process of Roquefort cheese (in which pure cultures are used) that any one

desiring a culture of this species can always obtain it by purchasing cheese of this type. It also occurs frequently in ensilage and is often found as a contamination in laboratory cultures of other fungi. The species is well marked by cultural characters from which it can be readily recognized when once studied. Its agency in the ripening of Roquefort, Gorgonzola, and Stilton cheeses is discussed in another paper (Thom²⁵).

CULTURAL DATA.

Color, gray-green to clear green, becoming brownish when old if exposed; reverse colorless or cream; color in media, none, or in some cases a slight and evanescent greenish tinge.^a

Odor, none (unless accountable for some odor in Roquefort cheese).

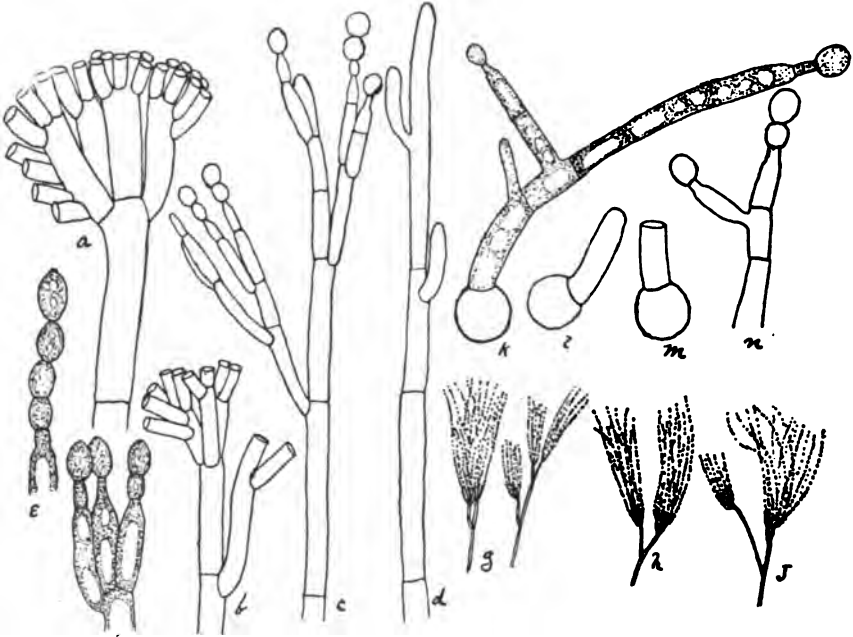


FIG. 4.—*Penicillium roqueforti* Thom: a, part of conidiophore and of base of fructification, highly magnified, showing production of basidia on sides as well as at apex of basidiophore; b, c, other types of branching; d, young conidiophore just branching; e, f, basidia and the formation of conidia, highly magnified; g, h, j, diagrams of types of fructification as seen under low power ($\times 80$); k, l, m, n, germination of conidia and new conidia produced directly on the first hyphae. (From Bulletin 82, Bureau of Animal Industry.)

Fifteen per cent gelatin in water, good growth, not heavy; liquefaction, none, or partial after 2 to 3 weeks in acidified cultures; litmus reaction, alkaline. Potato agar and bean agar, gray-green, loose, becoming dense and deep green when sugar is added. Potato plugs, characteristic. Raulin's fluid, very dense, deep green colonies. Cohn's solution, slight growth.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, slow growing but typical colonies up to 30 per cent, persistently green, not acid. Lactose 3 per cent, weak

^a Weidemann³⁵ records the production of a red color in cane-sugar solution by this species. The writer has not obtained this color in his cultures.

growth, evidently carbon deficiency. Lactic acid 0.9 per cent, better than lactose, not typical colony, good color. Levulose 3 per cent, weak colonies. Galactose 3 per cent, good growth, alkaline. Glycerin 3 per cent, weak growth. Alcohol, good growth, becoming brown in age. Tartaric acid, very slight growth. Butterfat, rich growth.

Milk, growth good, alkaline to litmus; curdling (0.25 per cent of calcium chlorid added) in 10 days; digestion, fairly rapid; color in milk, none.

At 37° C., no growth; grew when cooled; check at 20° C., good colony.

PENICILLIUM PURPUROGENUM O. Stoll.

Beitr. z. morph. u. biol. Char. *Penicillium*, Würzburg, 1904, p. 32, t. I, fig. 6; t. III, fig. 2; t. IV, fig. 3.

Colonies on lactose gelatin and potato or bean agar, gray-green to brown or olive, deeper green upon cane-sugar media, closely floccose, almost velvety in surface appearance, spreading slowly over the substratum and producing in the whole mass of medium a red color. In acid media rich in sugar secondary floccose mycelium arises white or with hyphæ studded with yellow granules. Conidiophores 100–300 by 3.5 μ , arising separately or from portions of hyphæ just above the surface of the substratum. Conidial fructification 50–100 μ in length, composed of one verticil of branches (sometimes with a secondary or partial secondary verticil), bearing whorls of conidiiferous cells 11–12 by 2.5 μ , narrowed abruptly to form sterigmata at the apices. Chains long, divergent. Conidia elliptical, 3.4–3.8 μ by 2–2.5 μ , green, granular, with from one to several small highly refractive granules in each, in chains falling apart in fluid mounts. Colonies liquefy sugar-gelatin slowly in 15 to 20 days.

Received from Kral in Prague.

The authority for the name of this species is attributed by Saccardo to Otto Stoll, as here indicated, since his description, or rather discussion, of this fungus forms the basis of Saccardo's Latin diagnosis. Stoll quotes the name from Kral, who gives the author as Alex. Fleroff, in Warsaw. Stoll has given the first discussion that is in any way adequate.

A closely similar organism has been found by Prof. F. D. Heald upon corn (*Zea mays*) in Nebraska. A third form corresponding closely in morphology and many cultural characters was sent from Miami, Fla., by Professor Rolfs. Although distinguishable by some characters, these forms resemble *P. purpurogenum* as described above so closely in morphology and cultural characters as to justify including them, temporarily at least, under this name. Neither of these forms produces the purple color as rapidly or as purely as the original race of *P. purpurogenum*.

CULTURAL DATA.

Color gray-green, becoming dark green with the presence of cane sugar; reverse yellow to reddish, or colorless; color in media, none to red to deep purple, almost black, according to medium; odor, none.

Fifteen per cent gelatin in water, medium growth, characteristic fruiting; liquefaction, partial in cultures 3 weeks old, or none, none in 10 to 12 days; litmus reaction, slowly alkaline or often neutral. Potato agar and bean agar, good colonies but no purple color, purple produced when sugar is added. Potato plugs, mycelium yellow

with granules, potato becoming purple. Raulin's fluid, slow development. Cohn's solution, germination only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar 10 per cent in agar, rich growth, deep red medium. Lactose 3 per cent, very little growth, very pale purplish. Lactic acid 0.9 per cent, medium growth, slowly producing purple in medium. Levulose 3 per cent, small colonies, fluid pale red. Galactose 3 per cent, small colonies, acid reaction. Glycerin 3 per cent, no growth. Alcohol 3 to 5 per cent in agar, good growth but slow, medium becoming purple. Potato starch 3 per cent, good growth, abundant purple in medium. Tartaric acid, no growth. Butterfat, very slow growth with purple color in fluid.

Milk, slow development; curdling (0.25 per cent calcium chlorid added) 13 days; digestion slow and only partial; color in milk, shades of purple according to progress, deepest under colony.

Cooked apple, feeble growth.

Grew equally well at 37° C. and 20° C.

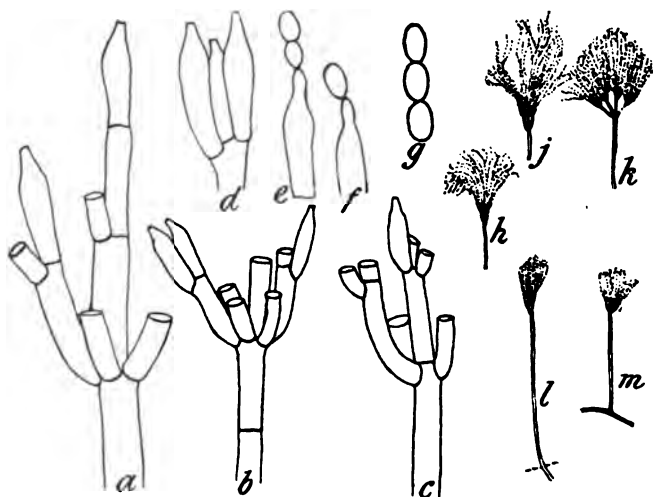


FIG. 5.—*Penicillium purpurogenum* O. Stoll: a, b, c, conidial fructification showing branching and arrangement ($\times 900$); a, form with partial secondary verticil; d, e, f, g, conidiiferous cells and conidia ($\times 1,400$); h, j, k, l, m, sketches of whole fructifications ($\times 140$).

***PENICILLIUM PINOPHILUM* Hedgcock (nomen novum).**

Syn. *Penicillium aureum* Corda, emended Hedgcock, Mo. Bot. Gard. Rept. 17, pp. 105–107, pl. ii, figs. 1–3.

Not *P. aureum* Corda, Prachtflora, p. 38, t. XVIII.

Colonies on potato or bean agar and milk sugar gelatin, from green on agar through shades of yellow-green to bright yellow and orange on media containing starch and cane sugar. Superficial hyphae studded with yellow granules upon acidified media. Reverse of colony and substratum (upon these media) colored deep rich red. Surface growth partly of simple conidiophores, partly aerial hyphae, and ropes of hyphae (which rarely become vertical coremia) bearing conidiophores as lateral branches. Conidiophores 100–200 μ . Conidial fructifications up to 120 μ in length, consisting of single verticils of branches 10–16 by 2–2.5 μ , bearing whorls of conidiiferous cells 13–15 by 2–2.5 μ tapering into acuminate sterigmata bearing conidial chains which are parallel but do not form a column. Conidia elliptical, 3–3.6 by 2 μ , smooth, pale green or yellowish green.

Colonies liquefy gelatin, but slowly and incompletely, and give a neutral or acid reaction upon all litmus media. Under different conditions of culture and acidity the discoloration of the medium varies from yellow to orange and deep red. Produces discolorations upon commercial timbers. Habitat, pine wood, which is strongly colored by it.

Culture received from the author, G. G. Hedgcock, of the Forest Pathological Laboratory, Bureau of Plant Industry, United States Department of Agriculture.

Since the publication of his description of this fungus (1906) Hedgcock⁹ has reached the conclusion, concurred in by the writer, that this species can not be regarded as identical with the species described by Corda as *P. aureum*. He notes that this species is a common agent in the discoloration of pine wood, hence proposes the name *P. pinophilum* (here first published). Careful consideration of Corda's figure and description would establish a strong presumption that

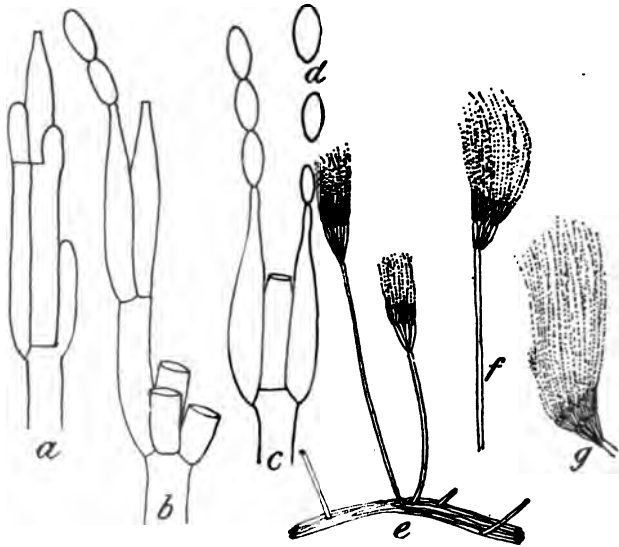


FIG. 6.—*Penicillium pinophilum* Hedgcock: a, young conidial fructification showing conidiiferous cells at apex of central branch before all the branches appear ($\times 1,600$); b, a verticil of four branches, upon one of which fruit appears ($\times 1,600$); c, d, conidiiferous cells and conidia ($\times 1,600$); e, rope of hyphae bearing conidiophores sketched ($\times 140$); f, g, forms of conidial fructification ($\times 140$).

the form described by him would not now be considered a species of *Penicillium*.

CULTURAL DATA.

Color, conidial areas green, vegetative mycelium colorless or studded with yellow granules; reverse of colony red; color in media, red.

Odor, none.

Fifteen per cent gelatin in water, growth slow, surface growth of conidiophores and green conidial fructifications only; liquefaction, none or very slow (in acidified cultures only after several weeks); litmus reaction, acid. Potato agar and bean agar, mycelium studded with yellow granules, conidial areas strict, green; reverse of colony, red. Potato plugs, poor growth, not typical. Cohn's solution, spores germinated only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, growth fair, hyphæ yellow with granules, acid reaction, conidia green, reverse, red. Lactose, growth slow, not characteristic. Levulose 3 per cent, fair growth, yellow mycelium, acid to litmus. Galactose 3 per cent, fair growth, acid reaction. Glycerin, very slight growth which became typical when sugar was added. Potato starch, good characteristic growth. Butterfat, slow weak growth, with characteristic colors and red fluid.

Milk, growth small; curdling (0.25 per cent calcium chlorid added), very slow; digestion, none or very slight; color in milk, red at top.

At 37° C., grew well, more rapidly than at 20° C.

PENICILLIUM RUBRUM O. Stoll.

Beitr. z. morp. u. biol. char. *Penicillium*, Würzburg, 1904, p. 35, t. I, fig. 7; t. III, fig. 3; t. IV, fig. 4.

Colonies upon lactose gelatin and potato or bean agar, from green through ochraceous to ochraceous red with varying conditions; consisting of green conidia with yellow mycelium when cane sugar is added. Aerial portion velvety strict or very closely floccose in media without sugar, becoming dense cushions of mycelium bearing successive crops of green conidia in cane-sugar media; marginal growth continuous but slow and not marked by a white border. Reverse and mycelium yellowish to red, yellow in sugar media; the substratum also colored in old agar colonies. Conidiophores arising from substratum directly or as very short lateral branches of the felted hyphæ, mostly 15-30 by 3-3.5 μ , swollen at the apex, making a dense layer on the surface. Conidial fructification usually massed into a heavy column with a broad triangular base, 100-200 μ in length, from a dense verticil of branches of the conidiophore, each swollen at the apex. Conidiiferous cells 10-13 by 2-3 μ , with rather abrupt points from which the conidia are cut off. Conidia at first cylindrical, then elliptical or even globose, 3.4 by 2 μ , or 2.5-3.3 μ , yellowish green to dark green when mature. Colonies produce slow and only partial liquefaction of sugar gelatin.

A slow-growing fungus fruiting for several weeks and differing greatly in colors with slight and undefined differences in the conditions. Sometimes producing a blood-red color on the reverse of the colony.

Cultures received from Kral. So far not found native in America.

PENICILLIUM LUTEUM Zukal.

Sitzber. K. Akad. Wiss. (Vienna) Math. Naturw. Kl., XCVIII, p. 521, 1889.

Conidial form: Colonies on sugar gelatin and potato or bean agar, white or gray or transiently yellow on media lacking sugars, with sometimes greenish areas of conidial fructification showing shades of yellow (egg yellow) upon sugar media, later passing over to reddish, especially with the formation of aerial wefts or balls of hyphæ producing asci (several weeks); surface rather close floccose, spreading indefinitely upon the substratum. Reverse of colony more or less reddish, especially on sugar media. Conidiophores a thin and incomplete layer, scantily produced mostly as lateral branches of aerial hyphæ, 20-100 μ (mostly 30-60) by 3 μ . Conidial fructification usually small up to 80 μ in length, commonly with a single lateral branch and but two verticils of long acuminate basidia 13-16 by 3-4 μ . Conidia elliptical to fusiform, 2.4 by 2.3 μ , rather firm walled, greenish, swelling greatly and producing 1 or 2 tubes in germinating.

This species characteristically produces yellow mycelium, from which, in a time varying from a few days upon media rich in sugar to several months upon plain potato agar, ascigerous wefts of hyphæ arise. As given by Wehmer,²⁹ "ascigerous conceptacles" are 0.5-2 mm. in diameter, globose, vitelline then red; asci reddish, globose to fusiform 8.8 by 7-7.8 μ ; sporidia 4.8 by 3.3 μ , transversely tricostrate, hyaline to

reddish. Colonies do not liquefy, or only slowly and partially liquefy, sugar gelatin. Litmus reaction neutral, the shades of purple found about the turning point of litmus. Colonies on apple are brighter yellow and produce ascigerous masses in very few days.

Received from Professor Thaxter, identified from the ascigerous form by Dr. C. Wehmer.

The author is convinced that Wehmer²² is in error in attributing a large degree of polymorphism to this fungus. Numerous cultures watched under all sorts of conditions are evidence that this species does not produce prominent coremia or large masses of green conidia.

CULTURAL DATA.

Color white, but mycelium studded with yellow granules in acid media, with sometimes reddish areas and conidial green areas; reverse of colony yellow to orange; color in media, pink in potato.

Odor, none.

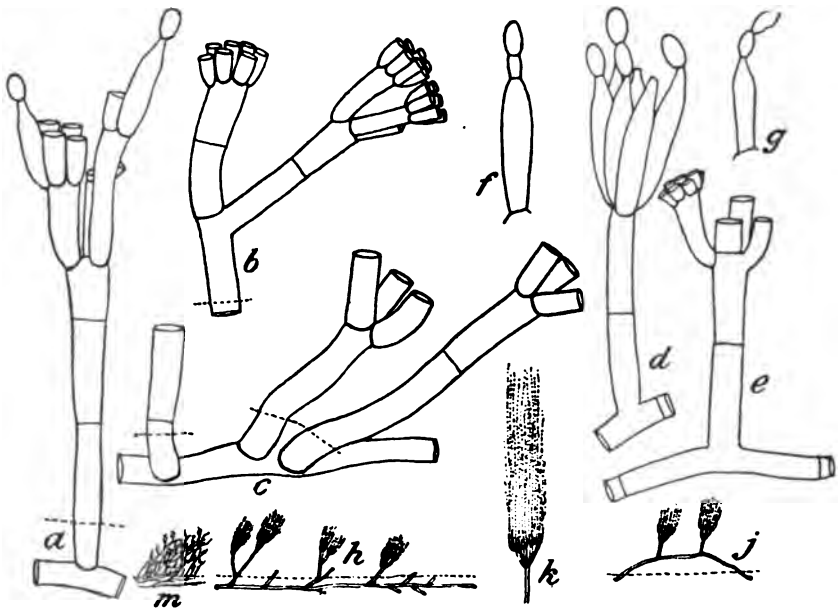


FIG. 7.—*Penicillium rubrum* O. Stoll: a, b, c, d, e, Whole conidiophores and the branching of conidial fructifications ($\times 1,400$); f, g, conidiferous cells and conidial formation ($\times 1,400$); h, j, sketch and diagram of habit of growth ($\times 140$); k, sketches of old conidial fructification in large size ($\times 140$); m, diagrammatic figure (the successive series of conidial fructifications are produced by new branches from hyphae overgrowing the earlier series). (Drawn from gelatin culture, but found with approximately the same morphology upon potato agar.)

Fifteen per cent gelatin in water, medium growth; liquefaction none; litmus reaction acid; potato agar and bean agar, mycelium transiently yellow, then colorless or reddish or yellowish gray; potato plugs, white to gray, in parts yellow, potato pinkish; Raulin's fluid, slow development, bright yellow colonies; Cohn's solution, germination only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, good growth, yellow, up to 30 per cent sugar, no growth at 50 per cent. Lactose 3 per cent, very slight growth; lactic acid 0.9 per cent, very slight growth. Levulose 3 per cent, very slight growth. Galactose 3 per cent, typical, acid reaction. Glycerin 3 per cent, germination only.

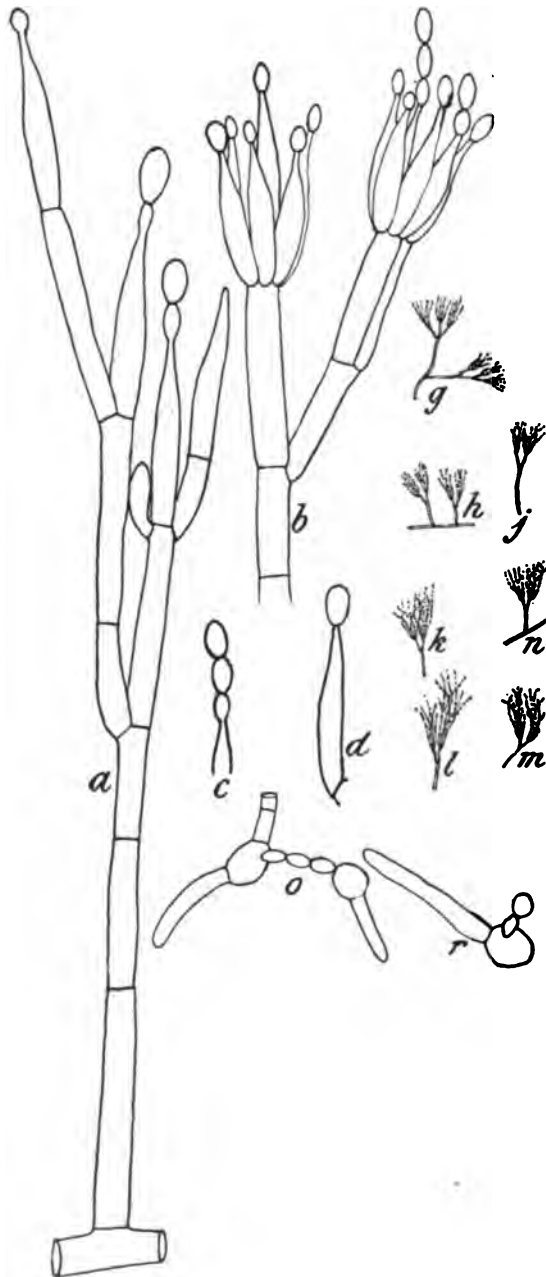


FIG. 8.—*Penicillium luteum* Zukal: *a*, whole conidiophore and fructification ($\times 900$); *b*, *c*, *d*, conidiferous cells, conidia and their arrangement ($\times 900$); *g*, *h*, *j*, *k*, *l*, *m*, *n*, sketches of fructifications ($\times 140$); *o*, *r*, swelling and germination of conidia, swollen conidia in the same chain with those which refuse to grow ($\times 900$).

Potato starch 3 per cent, slow development but characteristic. Butterfat, little or no growth.

Cooked apple, mycelium yellow, ascigerous masses produced most quickly (1 week).

Milk, grows poorly; curdling (with 0.25 per cent calcium chlorid) none; digestion slight and slow.

At 37° C., grew well; at 20° C., more slowly.

***PENICILLIUM DUCLAUXI* Delacroix.**

Bulletin de la Société Mycologique de France, Tome VIII, 1891, p. 107, Pl. VII.

Colonies grown upon gelatin and potato or bean agar clear dark green to olive when old, consisting of short crowded conidiophores arising for the most part singly from the substratum (strict), but sometimes producing short coremia. Long coremia are produced abundantly upon orange, milk, potato, and all media rich in cane sugar. Conidiophores very short, 10–50 μ , either arising directly from the substratum or borne

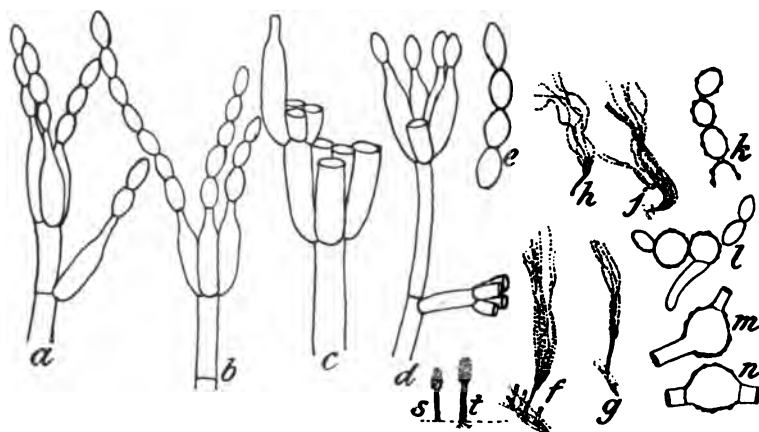


FIG. 9.—*Penicillium duclauxi* Delacroix: a, b, conidial fructifications with young conidia smooth, from potato-agar plate culture, simpler types ($\times 900$); c, d, e, conidial fructifications from potato-agar plate culture, more complex types ($\times 1,400$); f, g, h, j, sketches of habit upon potato agar, showing the very short conidiophores arising from the substratum ($\times 140$); k, ripe spores highly magnified to show delicate markings ($\times 900$, apochromatic); l, m, n, germination of conidia ($\times 900$, apochromatic); s, t, coremia, (sketch).

upon the upper third of the coremia, 1–2 septate, bearing a simple conidial fructification or a terminal fructification and a divergent lateral branch with a whorl of conidiiferous cells. Conidial fructification often 100–160 μ in length consisting of a few conidiiferous cells 10–12 μ in length in a simple terminal whorl or less commonly in secondary whorls. Conidia elliptical fusiform 3.6–4 by 2–2.5 μ , clear homogeneous green, smooth when young, but rugulose when ripe. Colonies liquefy sugar-gelatin in Petri-dish culture slowly from twelfth to twenty-fifth day and change red litmus to blue in 7 days. Produces a coloring agent in sugar media which is wine red in alkaline media and yellow (bile-yellow) in acid media (acts as an indicator with neutral point very near that of phenolphthalein).

This fungus is characterized by its enormous development of coremia upon milk, orange, apple, and media containing cane sugar, while producing only very short conidiophores in bean or potato agar and gelatin free from sugar.

Received from the author, George Delacroix, Paris, but one culture previously obtained from P. H. Rolfs in Florida in the summer of 1905 was thought to be this.

This species has not been found commonly in America, but it has been many times observed and recognized as a contamination of other cultures in this laboratory since its introduction. Its identification is therefore easy.

CULTURAL DATA.

Color, fruiting areas olive to clear deep green, often brown when old; reverse, from colorless to yellow and shades of red, according to medium; color in media, none in media free from sugar, in sugar yellow at first, then slowly red (color acts as an indicator—acid when yellow, alkaline when red).

Odor, rather strong in Raulin's fluid, none in most cultures.

Fifteen per cent gelatin in water, medium growth; liquefaction, none in 15 days, then slow in acidified cultures especially; litmus reaction, neutral slowly or weakly alkaline. Potato and bean agar, green fruiting surfaces consisting of very short crowded conidiophores slightly tuberculate at times, but no coremia. Potato plugs, abundant coremia, deep green, reverse and potato yellowish. Raulin's fluid, good growth, many coremia, some odor. Cohn's solution, germination only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar 3 per cent, rich growth, coremia, poor colonies at 30 per cent and 60 per cent. Lactose 3 per cent, very weak growth, few very small coremia. Lactic acid 0.9 per cent, good growth, typical, coremia many, remains acid. Levulose 3 per cent, weak colonies. Galactose 3 per cent, poor growth. Glycerin 3 per cent, germination and slow, weak colonies. Alcohol, some growth. Potato starch, rich growth, coremia, reverse and fluid yellowish. Butterfat, slow but typical coremiform colony, fluid yellow (acid reaction).

Milk, typical coremiform colonies; curdling (0.25 per cent calcium chlorid added) in 9 days; digestion slow; color, becoming yellow (acid) and later red (alkaline) in very old cultures.

At 37° C. no growth; check grew at 20° C.

PENICILLIUM CLAVIFORME Bainier.

Bulletin Trimestriel de la Société Mycologique de France, Tome XXI, 1905, p. 127, Pl. XI, figs. 8–11; Saccardo, Sylloge Fungorum, Vol. XVIII, p. 520.

Colonies on milk-sugar gelatin and potato agar, white or gray, with surface composed of loosely floccose hyphæ, bearing simple but definitely penicillate fructifications, between the bases of white or yellowish simple or variously branched coremia 1–2 cm. long, fertile only at the apices. Simple fructifications sparingly branched, bearing small verticils of conidiiferous cells 9–10 by 2 μ . Coremial fructifications consisting of closely branching and interwoven hyphæ, producing verticils of conidiifer-

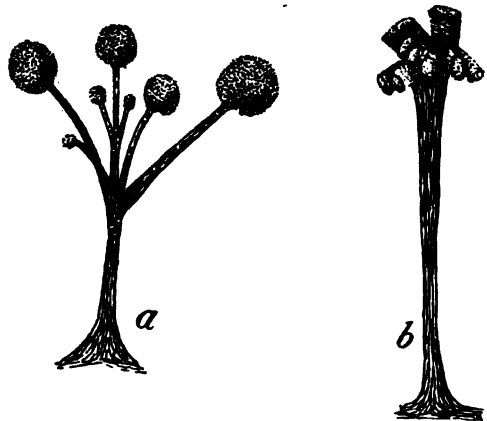


FIG. 10.—*Penicillium claviforme* Bainier: a, coremium grown upon sugar media, showing branching stalk with several small heads of conidia; b, coremium grown upon gelatin free from sugar, showing typical unbranched stalk with a single conidial mass splitting as it increases in size into several columns composed of chains of conidia. (For full illustration of the structure of this species, see Bainier's figures.)

ous cells crowded into a false hymenium and producing chains of conidia adhering in olive-green masses 1-3 mm. in height. Conidia elliptical, showing a connective, 4.2-4.6 by 3-3.3 μ , homogeneous green, remaining in chains in fluid mounts. Colonies only partially liquefy gelatin media and give a weak alkaline or neutral reaction with litmus.

Received from G. Bainier October, 1905.

A culture received from Reddick, Ithaca, N. Y., marked Whetzel No. 2095, proved to be this species. It was found at Junius, N. Y. A culture sent to Dr. C. H. Peck was not recognized. Although not closely resembling other species of *Penicillium* it may best continue under the name given by Bainier until closer affinities are found for it.

CULTURAL DATA.

Color, mycelium white or gray, conidial heads olive green; reverse of colony brown; color in media brownish.

Odor, perceptible in media containing cane sugar, characteristic.

Fifteen per cent gelatin, growth, characteristic coremia, but mainly unbranched; liquefaction slow, partial; litmus neutral. Potato agar and bean agar, typical coremiform colonies. Potato plugs, vigorous typical growth with long coremia. Raulin's fluid, characteristic. Cohn's solution, characteristic.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, typical growth with acid reaction in concentrations from 1.5 to 30 per cent, coremia branching, no coremia at 50 per cent. Lactose 3 per cent, slight growth, poorly nourished colonies, very delicate coremia. Lactic acid 0.9 per cent, germination only. Levulose 3 per cent, slow-growing, poorly nourished colony. Galactose 3 per cent, typical growth, acid reaction. Glycerin 3 per cent, not typical, no coremia, coremia produced after addition of cane sugar. Malic acid 1 per cent, slight growth, few and very small coremia. Butterfat, typical colonies.

Milk, good characteristic growth; curdling (0.25 per cent calcium chlorid added) in 9 days; digestion slow but fairly complete; color in milk brown or reddish.

At 37° C., no growth in 6 days; culture grew when cooled to 20° C.

PENICILLIUM GRANULATUM Bainier.

Bul. Soc. Mycol. France, XXI, 1905, p. 127, Pl. XI, figs. 6, 7.

Colonies upon plain gelatin and potato or bean agar yellowish green to gray or grayish brown, superficially composed of crowded small coremia 1-3 mm. in height, mixed with floccose hyphæ and separate conidiophores, spreading indeterminately upon the substratum. Reverse reddish orange (approaching "fulvous"), aerial hyphæ delicately granular or spinulose, which separates this from all other species studied. Conidiophores 4-4.5 μ in diameter, short or very long, either separate or, mostly, massed into very short, crowded coremia (less than 1 mm. in height). Conidial fructifications usually 100-200 μ in length, once or twice verticillate, with many conidiiferous cells 9 by 2-2.5 μ , and long, loosely divergent chains of conidia. Conidia at first cylindrical, then elliptical to globose, about 2.5-3 by 3-3.5 or 3 μ in diameter, yellowish green, granular, remaining in long chains in fluid mounts. Colonies do not liquefy gelatin, litmus reaction slowly alkaline.

The delicately granular or spinulose hyphæ as noted and figured by Bainier are a valid and distinctive character. The species is also easily recognized by its general appearance and habit. Obtained from the type cultures of Professor Bainier, Paris.

CULTURAL DATA.

Color yellowish green to green and later various shades of brown; reverse of colony orange, or yellow to deep orange, or even red; color in media yellow to orange to red in media containing starch or sugar.

Odor, none.

Fifteen per cent gelatin in water, characteristic growth; liquefaction, none, or partial after several weeks in acidified cultures; litmus reaction, strongly alkaline. Potato agar and bean agar, yellow-green to brown, yellow below, coremia not closely crowded as in sugar media. Potato plugs, characteristic growth, potato stained yellow to deep brownish yellow. Raulin's fluid, characteristic growth. Cohn's solution, germination only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, 3-30 per cent, characteristic growth and colors, but not normal at 60 per cent, with acid reaction. Lactose 3 per cent, characteristic structure but weak development. Lactic acid 0.9 per cent, growth, but not normal colonies. Levulose 3 per cent, slow development, small colonies. Galactose 3 per cent, characteristic. Glycerin 3 per cent, slight growth, grew freely when cane sugar was added. Potato starch, characteristic. Butterfat, typical growth, reverse and fluid orange; yellowish.

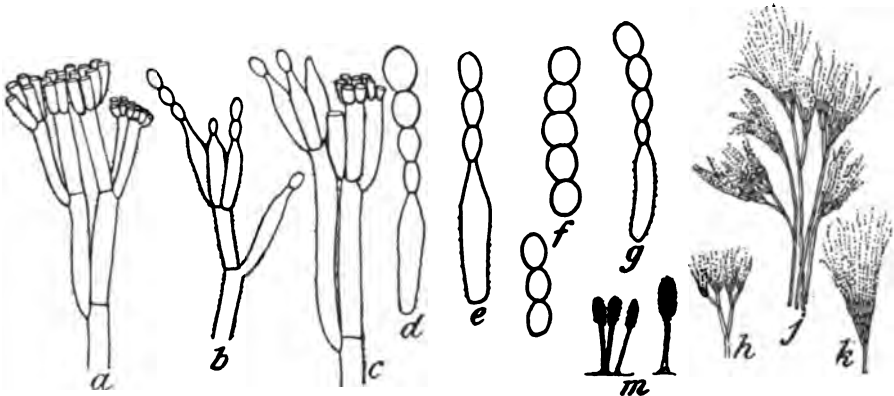


FIG. 11.—*Penicillium granulatum* Bainier: a, b, c, branching of conidial fructification showing granulated echinulated cell walls ($\times 900$); d, e, f, g, conidiiferous cells and conidial chains ($\times 1,400$); h, j, k, sketches of fructifications ($\times 140$); m, sketches drawn from photomicrograph of coremia showing fertile and sterile areas ($\times$ about 25).

Milk, rapid and characteristic growth; curdling, none; digestion, slow but complete (fairly); color in milk, yellow to orange to deep red.

At 37° C., no growth, conidia grew when removed to lower temperature; at 20° C., excellent growth.

PENICILLIUM BREVICAULE Saccardo.

Fungi Italici, No. 893, Mich., II, p. 547.

Colonies grown upon sugar gelatin grayish white, then yellowish brown or chocolate, consisting of short closely crowded conidiophores making powdery areas overgrown by loosely trailing floccose hyphæ and ropes of hyphæ, with broadly spreading indeterminate margin. Coridiophores, short, $10-30\mu$ mostly, arising directly from the submerged hyphæ, or numerous and irregularly borne as lateral and perpendicular branches of trailing aerial hyphæ and ropes of hyphæ. Conidial fructifications either simple chains terminating unbranched or sparingly branched conidiophores in young colonies, or verticillately and irregularly twice verticillately branching systems bearing numerous divergent chains often 150μ in length in old colonies. Conidiiferous

cells continuous with conidiophores 12–15 by 4μ tapering to slender sterigmata. Conidia somewhat pear-shaped, slightly tuberculate at apex, with broad base, $6.5\text{--}7.5$ by $7.5\text{--}9\mu$, in mass light brown to chocolate; at first smooth, then with thick tuberculate walls, viable for many months, germinating by a single tube from the thin center of the broad base into a bulbous enlargement from which mycelial hyphae about 2μ in diameter arise. Mycelium very thin walled, narrow cells of varying length. Colonies liquefy sugar gelatin and give a strong blue reaction in litmus media, but grow very tardily, if at all, in potato or bean agar. Grows very rapidly upon neutral or alkaline media, but very slowly or not at all in media acid to litmus. Digests milk. Refused to grow after repeated inoculation into sterilized apple. Spores which refused to germinate in the agar media used grew immediately when transferred to any of the gelatin media.

Cosmopolitan, forms characteristic chocolate patches on Camembert cheese. Secured from numerous brands of cheese and common in the laboratories of this station.

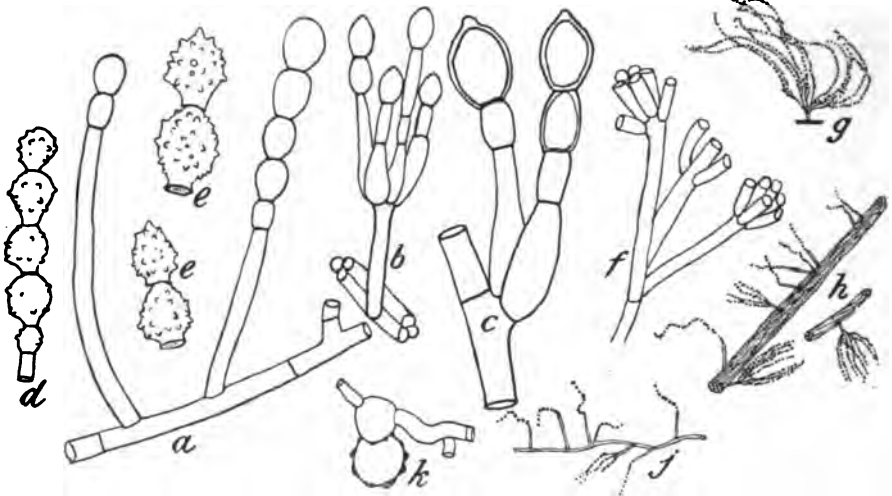


FIG. 12.—*Penicillium brevicaulis* Saccardo: *a*, conidiophores and simple conidial chains with spores still smooth ($\times 900$); *b*, *f*, more complex conidial fructifications ($\times 900$); *c*, two young conidial chains, showing thick walls of spores ($\times 1,400$); *d*, *e*, conidia after becoming echinulate ($\times 1,400$); *g*, *h*, *j*, sketches of forms and habit of conidial fructifications ($\times 140$); *g* from an old culture, sessile or almost so; *h* and *j* show tralling hyphae and a rope of hyphae with lateral conidiophores; *k*, germinated conidium where the old spore wall lies empty beside the growing cell ($\times 1,400$).

The author does not believe that this species is closely related to other species of this genus, but since it has been placed here by a very liberal interpretation of descriptions it may perhaps remain under this name until someone finds out its real affinities. The two forms which follow as varieties are found in the same habitat, show closely similar morphology, and give almost identical physiological reactions. Their designation as varieties of *P. brevicaulis* may therefore be justified, though one at least (var. *glabrum*) seems separate enough to warrant proposing for it a specific name.

CULTURAL DATA.

Color, conidial surfaces clay-yellow to chocolate; reverse of colony, mycelium colorless; color in media, none.

Odor, ammoniacal, used as a test for arsenic (Gosio et al.).

Fifteen per cent gelatin in water, typical colony; liquefaction, rapid—5-6 days; litmus, strongly alkaline. Prefers alkaline, neutral, or only slightly acid media. Potato agar, spores sometimes refuse even to germinate, but grow when transferred to gelatin; grows poorly on agar media free from sugar, or peptone, or the by-products of other fungus growth. Common as a secondary growth in such plates. Potato plugs, good colonies. Cohn's solution, characteristic but slow growth. Raulin's fluid, germinated, but very slight growth.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar in agar 3 per cent, failed to grow, same spores transferred to gelatin media grew promptly. Lactose 10 per cent, slow, characteristic color to conidia. Lactic acid 0.9 per cent, germination only. Levulose 2.5 per cent, growth characteristic, alkaline reaction to litmus. Galactose 3 per cent, characteristic growth, alkaline reaction to litmus. Glycerin 3 per cent, spores germinated only, grew well upon addition of cane sugar. Potato starch grew well. Malic acid, germinated only. Butter fat, slow development, finally producing drops of yellow oil which separate out.

Milk, rapid growth; curdling (0.25 per cent calcium chlorid added) in 10 days; digestion, rapid; color in milk, none.

Cooked apple, failed to grow after repeated inoculation.

At 37° and 20° C., grew equally well.

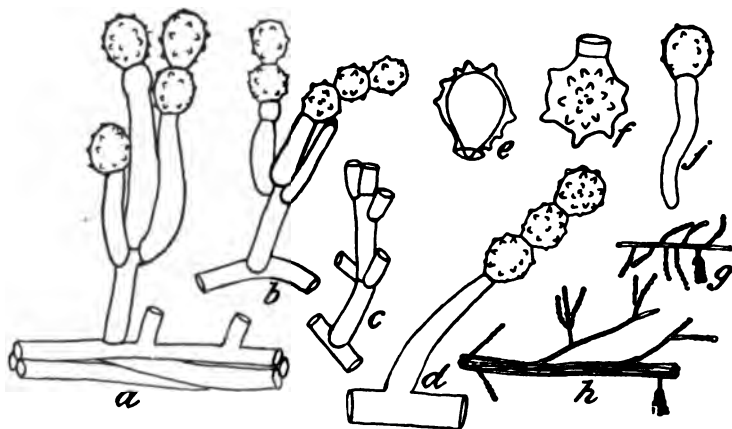


FIG. 13.—*Penicillium brevicaule*, var. *album*: a, b, c, d, conidiophores and fructification borne variously and differently branching ($\times 900$); e, f, ripe conidia; g, h, sketch of single hyphae and a rope of hyphae bearing conidiophores ($\times 140$); j, germinated spore.

PENICILLIUM BREVICAULE Saccardo, var. ALBUM Thom, n. var.

Colonies upon sugar gelatin white to cream-colored alike above and below upon all media, strict to sparsely floccose, with trailing hyphae and ropes of hyphae, indeminately spreading. Conidiophores either arising from substratum directly or mostly as perpendicular branches of aerial hyphae and ropes of hyphae 15-40 μ in length, conidial fructification varying from a single chain to more or less complex penicillate branching, mostly producing few chains of indefinite length and arrangement from narrow tapering conidiiferous cells. Conidia pyriform to subglobose, with basal collar, 9-10 μ , roughly tuberculate, white or slightly yellowish tinged, thick walled except at the base, the center of which remains as a germ pore. Colonies rapidly liquefy sugar gelatin with strong ammoniacal odor, and give an intensely alkaline reaction in litmus media. Gives exactly the same reactions as *P. brevicaule* Sacc. Differs from the latter slightly, except in the color of the spores.

Common upon imported Camembert cheese. Found often upon domestic Camembert and grows very readily in cheese cellars, where it becomes a nuisance.

CULTURAL DATA.

Same as *P. brevicaulis* Sacc., except the following difference noted:

Conidia cream, somewhat larger than *P. brevicaulis*.

Cohn's solution failed to produce characteristic colonies.

In Dox's solution, with butterfat as a source of carbon, it differs from *P. brevicaulis* by failing to cause drops of yellow oil to separate out.

Agar-agar: In repeated cultures this organism has failed to grow well in agar media. In some such cases the spores transferred from the agar to gelatin grew at once. Some cultures upon agar grow slowly and in typical manner, but the development upon all agar media has seemed uncertain. In synthetic solution cultures were obtained when the inoculation of tubes of the same solution with 1.5 per cent of agar added to make a solid substratum produced no growth.

***PENICILLIUM BREVICAULE* Saccardo, var. *GLABRUM* Thom, n. var.**

Colonies white or only slightly yellowish-tinged in all gelatin media, grow not at all or with difficulty on agar of most formulæ. Aerial portion consisting of short, closely crowded conidiophores making a powdery surface overgrown by loosely trailing hyphæ and ropes of hyphæ, spreading broadly over the substratum. Conidiophores, short, mostly 10–30 μ , arising directly from submerged hyphæ or numerous and irregu-

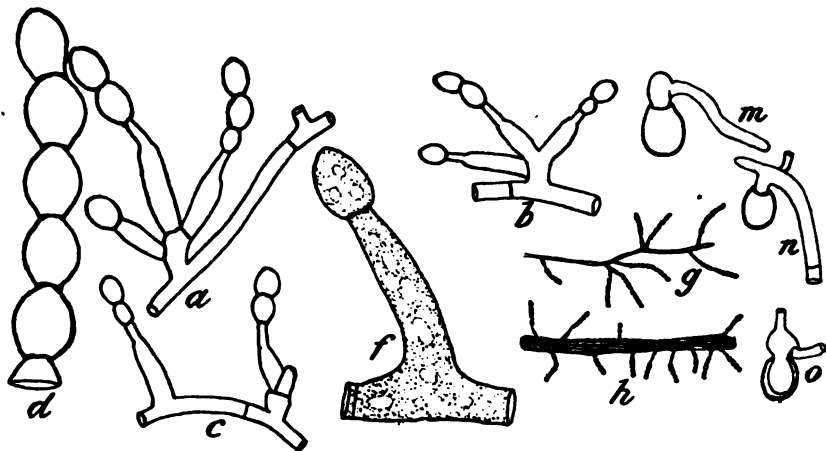


FIG. 14.—*Penicillium brevicaulis*, var. *glabrum*: a, b, c, branching of conidial fructification ($\times 900$); d, chain of conidia ($\times 1,400$); f, formation of conidium on young branch ($\times 1,400$); g, h, sketch of appearance in culture ($\times 140$); m, n, o, germination of conidia.

larly borne as perpendicular branches of the superficial hyphæ and ropes of hyphæ. Conidial fructifications from simple chains of spores to fairly complex penicillate groups of branchlets resembling *P. brevicaulis*, but mostly less complex. Conidia obovate, pyriform 7–8 by 8–10 μ or almost globose, 7–9 μ , smooth, white, rather thick-walled and retaining their power to germinate for many months. In old potato and other cultures black sclerotia are formed in the substratum but do not produce asci. Liquefies gelatin rapidly (within one week), gives a strong alkaline reaction and ammoniacal odor.

Habitat, found repeatedly on imported Camembert cheese and secondarily upon domestic soft cheese, where it grows into prominent cottony patches indistinguishable to the eye from the white variety of *P. brevicaulis*. This fungus is separated from *P. brevicaulis* by its smooth white spores and the production of the black sclerotia in the substratum.

This form is certainly closely related to *P. brevicaulis* by its physiological reactions and its general morphology. It was found in one case among the exsiccati in the Harvard herbarium under the name of *Monilia candida*. There is, however, no possibility of confusing this form with that species as understood and described by more recent students, such as Hansen and Jorgenson.

CULTURAL DATA.

Exactly as in *P. brevicaulis*, except for the following:

Conidia smooth, somewhat smaller.

Color more nearly white.

Sclerotia black, found in very old potato-plug cultures or in agar cultures which have grown several weeks or months.

Cohn's solution failed to produce a characteristic colony.

PENICILLIUM ROSEUM Link (?).

Colonies on milk-sugar gelatin or potato agar white to pink or salmon in fruiting areas, loose floccose with simple hyphæ and ropes of hyphæ, producing dense irregular

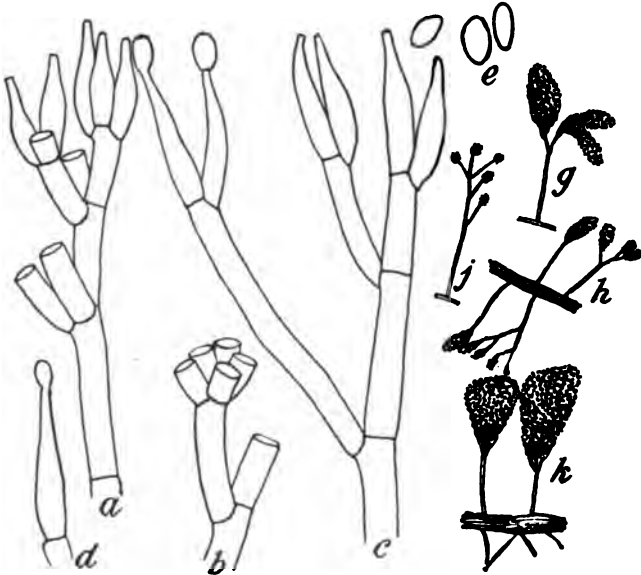


FIG. 15.—*Penicillium roseum* Link (?): *a*, *b*, *c*, branching of conidial fructification, showing few cells in each verticil ($\times 900$); *d*, *e*, conidiiferous cell and conidia ($\times 900$); *g*, *h*, *j*, *k*, sketches of ripe fructifications showing agglutination of conidia into slimy masses ($\times 140$).

pinkish masses or sclerotia up to 1 mm. or more in diameter in old culture. Conidiophores borne as perpendicular branches of aerial hyphæ or ropes of hyphæ, 45–125 μ . Conidial fructification up to 140 μ in length, once or twice irregularly alternately or verticillately branched, with conidiiferous cells varying from 12 by 2–3 μ in the verticils of 5 or less to 17 by 2.3 μ when solitary, bearing conidia which become aggregated into gelatinous balls or masses. Conidia colorless (pink or rosy in mass), elliptical, 5–7 by 3–5 μ , slightly apiculate, smooth, appearing delicately granular within. Colonies liquefy gelatin cultures rapidly and give an alkaline reaction to litmus media.

Brought from Kral, in Prague, Bohemia.

The same organism has been found once in accidental culture in this laboratory; received once from a correspondent in Halle, Germany, and later found under this name as No. 1179 in De Thümen's *Mycotheca Universalis*; collected by Ravenel in South Carolina in 1876 upon leaves of *Buxus*; this and several other specimens were found in the mycological collection of the Bureau of Plant Industry, United States Department of Agriculture. The spores are the same length as given by Saccardo, but slightly broader. The number of specimens found under this name from widely different workers appears to justify the belief that this is the organism described by Link under this name. If the development of a mucilaginous mass enveloping the conidia be regarded as a sufficient basis for separation of such species under the generic name of *Gliocladium*, this species would become *Gliocladium roseum* (Link).

The form upon *Buxus* is cited by Saccardo, referring to it as "*P. roseum* Cooke, non-Link," and held to be *Verticillium buxi* Auersw. et Fleisch. Examination of the material would indicate that in De Thümen's collection at least this species is more closely allied to the other species of *Penicillium* than to *Verticillium*.

CULTURAL DATA.

Color white or shades of salmon pink; reverse cream or white; color in media, none.

Odor, none.

Fifteen per cent gelatin in water, medium growth, white. Liquefaction, rapid. Litmus reaction, alkaline, strongly. Potato agar and bean agar, good growth, but white. Potato plugs, white colonies. Cohn's solution, slight growth.

In Dox's solution, with butterfat as a source of carbon, this species caused drops of yellow oil to separate out.

At 37° C., killed; check at 20° C., good.

PENICILLIUM CAMEMBERTI Thom.

Emended from U. S. Department of Agriculture, Bureau of Animal Industry, Bul. 82, p. 33, fig. 1, 1906.

Possible syn.: *P. album* Epstein (not Preuss), Archiv f. Hyg., Bd. 45, Hft. 4, p. 360, 1902.

P. epsteini Lindau, Deutschl. Krypt. Flora, Pilze, VIII, p. 166.

Colonies on potato agar or lactose gelatin effused; white (sometimes yellowish white), changing in 5-8 days to gray-green (glaucous); surface of colony floccose, of loosely felted hyphæ about 5 μ in diameter, reverse of colony yellowish white; conidiophores 300-800 μ in length, 3-4 μ in diameter, septate, cells thin-walled, often collapsing in age, arising as branches of aerial hyphæ; fructification sometimes 175 μ in length, but usually much less, consisting commonly of one main branch and one lateral branch, sparingly branched to produce rather few conidiiferous cells which bear long loosely divergent chains of conidia. Conidiiferous cells 8-11 by 2.4-3 μ . Conidia at first cylindrical, then elliptical, and finally globose when ripe, smooth bluish green by transmitted light, thin-walled and commonly guttulate, 4.5-5.5 μ in diameter, swelling in germination to 8-10 μ . Germ tubes one to several. Cells of mycelium about 5 by 20-40 μ . Liquefies lactose gelatin only under center of colony. Produces a strong

alkaline reaction in gelatin, free from sugar, but in sugar media produces a more or less persistent acid reaction. Growing and fruiting period, about 2 weeks. Fruits only upon exposed surfaces of the substrata; never produces spores in cavities not broadly open.

Habitat, Camembert and other soft cheeses.

The name *P. album* Epstein, which is also *P. epsteini* Lindau,¹⁰ is inserted in the list of possible synonymy because this name is accepted for this mold by Mazé¹³ in a recent paper. If we base identification upon the cultural characters given for his mold by Epstein, it could not have been *P. camemberti*. The characterization, so far as given by Epstein and extracted from his paper by Lindau, might refer to the pure white form (*P. candidum*) as interpreted by Roger and Mazé better than to this species. There is certainly at least a varietal difference (spore color) between *P. camemberti* and the *P. candidum* of Roger and Mazé, which is designated a variety of *P. camemberti* in this paper.

The change in litmus reaction in this description from that previously published is result of proof that no acidity is produced by this species except when sugar is present. A change from blue to red and black to blue indicates the production of acidity by fermentation of sugar followed by the neutralization of the acid so produced by the alkaline by-products of proteolysis or by a further change in the acid. Therefore this statement is best omitted from the diagnostic description.

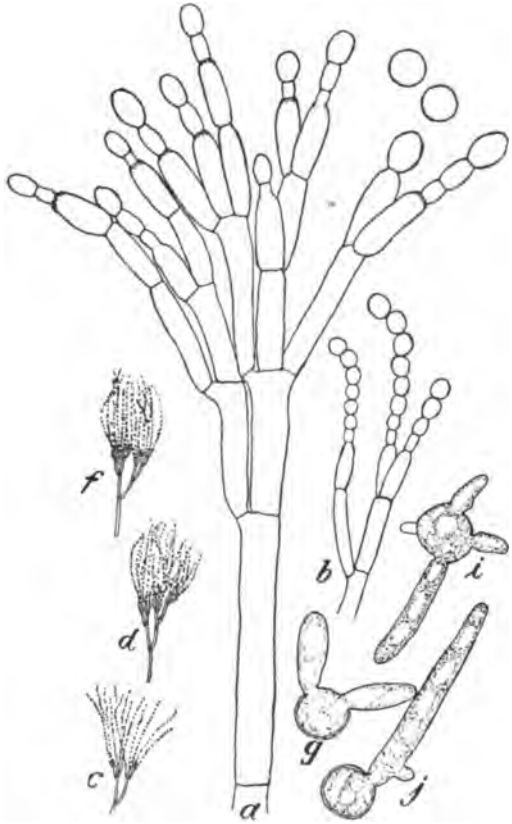


FIG. 16.—*Penicillium camemberti* Thom: a, conidiophore showing a common type of branching and the production of basidia and conidia, highly magnified; b, a common form showing much less branching; c, d, f, diagrams of large fructifications ($\times 80$); g, i, j, germinating conidia. (From Bull. 82, Bureau of Animal Industry.)

CULTURAL DATA.

Color white, through cream to a shade of gray-green upon sugar media, without sugar gray or drab, all becoming drab when old (exposure to light); reverse of colony, uncolored, cream; media, uncolored.

Odor, none, except in very old cultures upon milk or cereal.

Fifteen per cent gelatin in water, moderate growth, not rich; liquefaction, none, or slow and partial after 2 to 3 weeks in acidified cultures; litmus, strongly alkaline; colonies slow, moderate growth, typical. Potato agar and bean agar and potato plugs, fair, white to gray colonies lacking or nearly free from green color. Raulin's fluid, very heavy growth, white through pronounced cream to green shades. Cohn's solution, slow growth, characteristic, but lacking in green color.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, characteristic, abundant green color in solutions as high as 50 per cent, acid. Lactose 3 per cent, characteristic, acid reaction. Lactic acid 0.9 per cent, slowly typical, with ultimately an alkaline reaction to litmus. Levulose 3 per cent, slowly typical. Galactose 3 per cent, characteristic growth, very slowly developing the typical green color. Alcohol about 5 per cent, characteristic. Potato starch, characteristic. Malic acid 1 per cent, fair colony, colorless. Butterfat, slowly typical colony with a tinge of violet in reverse, no color in fluid.

Milk, typical growth; curdling (0.25 per cent calcium chlorid added) in 10 days; digestion, slow but fairly complete; color in milk, none.

Camembert cheese, capable of reducing moist curd soured by lactic organisms to a semiliquid condition.

At 35° to 37° C., no growth; at 20° C., excellent.

***PENICILLIUM CAMEMBERTI*, var. *ROGERI* Thom, n. var.**

Syn. *P. candidum* of Roger and Mazé, not Link.

Colonies grown upon sugar gelatin or bean or potato agar pure white, loosely and evenly floccose to the very margin, where aerial and submerged hyphæ grow with equal rapidity. Reverse of colonies white or yellowish white (not discolored). Conidiophores 3-5 by 100-800 μ varying greatly, mostly branches of aerial hyphæ. Conidial fructification 70-90 μ in length, loosely and irregularly branched and bearing rather few basidia at unequal heights, with divergent chains of colorless conidia. Branching system of conidial fructification sometimes 75 μ in length. Conidia smooth, hyaline, 4-4.5 or even 5.5 μ in diameter, globose or nearly so when ripe. Sugar gelatin is slowly liquefied under the center of the colony only, colonies never floating in a pool of liquid. Reaction in the medium is acid to litmus at first, then changes to alkaline.

This fungus has been found by the author only upon Camembert, Brie, and Neufchâtel cheeses from western Europe.

This variety has been discussed by Mazé¹³ as *P. candidum* Link. This seems an impossible application of the name *P. candidum* from Link's description¹² or that given by Saccardo, since the spores are stated to be 2-3 μ in diameter. Further, in such identifications no account is taken of a paper by Morini,¹⁸ in which an ascigerous stage is described for *P. candidum* Link. Under four years of cultivation no signs of an ascigerous form have been produced. Stoll²⁴ has considered *P. candidum* to be only a colorless *P. glaucum*, but as the author has so far failed to find a worker who will undertake to limit the name

P. glaucum to a special form, this does not mend matters. Long cultivation does show, however, that this organism is closely related to the one already described as *P. camemberti*.²⁵ Since this is the form given prominence in cheese studies by the work of Georges Roger,²¹ it seems most natural to regard it as a variety of the former species and designate it by Roger's name.

In Lafar's *Handbuch der Technischen Mykologie*, Professors Weigmann³⁶ and Wehmer³⁴ refer to this fungus as probably iden-

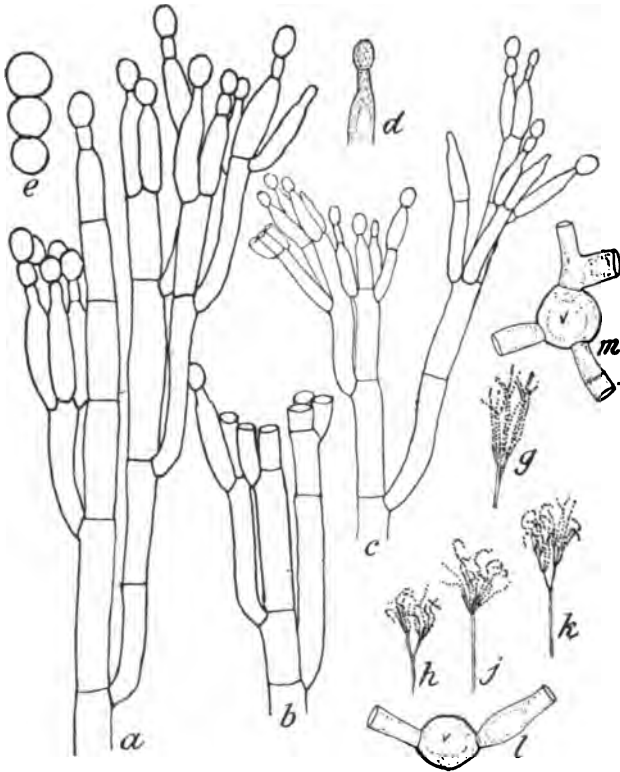


FIG. 17.—*Penicillium camemberti*, var. *rogeri*: a, b, c, types of branching of conidial fructification ($\times 900$); e, ripe conidia, showing variation in size ($\times 900$); g, h, j, k, sketches of conidial fructifications ($\times 140$); l, m, germination of conidia by several tubes.

tical with *P. camemberti*. However this may appear from examination of the literature alone, no one actually familiar with the cultures will claim such identity. If the possession of colorless spores be regarded as a case of albinism, this form may perhaps be regarded as a variety of *P. camemberti*. It has been kept separate and remained constant in culture for several years. It would seem, therefore, equally proper to regard it as a different species were it not so closely associated with *P. camemberti* in every other character.

The name *P. candidum* was used by Link for a mold growing upon decaying leaves, bulbs, and fungi, which was said to be common, and Morini¹⁶ later describes an ascigerous form of this fungus. The spores are of different size (2–3 μ). There appears to be no justification for adopting this name for the fungus used by Roger in cheese making.

CULTURAL DATA.

Different from *P. camemberti* only as follows:

Color of conidia persistently white.

Cohn's solution, failed to germinate. Same spores transferred to gelatin after 4 months grew normally.

Camembert cheese, does not produce the same texture as the preceding species.

PENICILLIUM BIFORME n. sp.

Latin diagnosis.^a—Coloniis in gelatina cultis, albis, lente glaucescentibus, densius floccosis, margine sterili lata, aut, in agar solani tuberosi cultis, albis glaucescentibus, mox avellaneis vel fere olivaceis, parte aëria ex conidiophoris brevissimis et creberrimis fructibus conidicisque composita; conidiophoris (sine ramis) 60–150 μ in agar, vel longioribus ramosis ex hyphis floccosis in gelatina cultis; fructibus conidicis 100–200 μ longis, plerumque 1–2 ramosis alternates, ramis convergentibus vel divergentibus, ramulis verticillatis basidia apice verticillata gerentibus; basidiis 8–10 usque 13 $\times$ 3 μ , conidiis primum ellipticis vel cylindricis demum globosis, 3.2–3.5 $\times$ 4–4.3, vel 4 μ diam., in catenis manentibus submersis; coloniis copiosis in saccharo lactis, gelatinam in parte lente liquefacientibus, alkalinis lacmo, odore mucidis.

Habitat, in caseo, ex Gallia.

Cultivated in gelatin, white, slowly gray-green, densely floccose, with broad vegetative margin, spreading widely over the substratum; in potato agar white, then gray-green, rapidly becoming yellowish-brown, drab, or almost olive, restricted in growth, aerial portion consisting of very short densely crowded conidiophores and conidial fructifications; conidiophores 60–150 μ on agar or slightly longer when arising as branches from the floccose aerial mycelium growing upon gelatin; conidial fructifications mostly once or twice alternately branched, branches convergent or divergent, each bearing a verticil of branchlets crowned by verticils of conidiiferous cells with chains of conidia, the whole 60–240 μ , usually 100–200 μ , in length; conidiiferous cells 8–10 or even 13 by 3 μ ; conidia elliptical or cylindrical, then globose, 3.2–3.5 by 4–4.3 μ or 4 μ in diameter, adhering in chains in fluid mounts; grows luxuriantly in fluid offering milk-sugar as source of carbon, partially and slowly liquefies gelatin, with alkaline reaction to litmus; odor, very strong, "moldy," characteristic.

This species was obtained from cheese sent from France by Georges Roger. It was afterwards obtained from other French cheeses, but does not seem to have any economic importance. It is closely related by cultural characters as well as morphology to *P. camemberti*, with which it shares the ability to grow normally in fluid offering lactose as the source of carbon, but differs in its short conidiophores, diverse habit upon potato agar and gelatin, and its strong charac-

^a The author is indebted to Prof. H. R. Monteith, of the Connecticut Agricultural College, for much assistance in preparing the Latin diagnoses.

teristic smell. It is perhaps intermediate in character between *P. camemberti* and the group of forms designated as *P. commune* in this paper.

CULTURAL DATA.

Color, white to gray-green, gray, or drab in some media, all brown or drab when old; reverse cream; color in media, none.

Odor, very strong peculiar "moldy" odor, typical of this species under nearly all conditions.

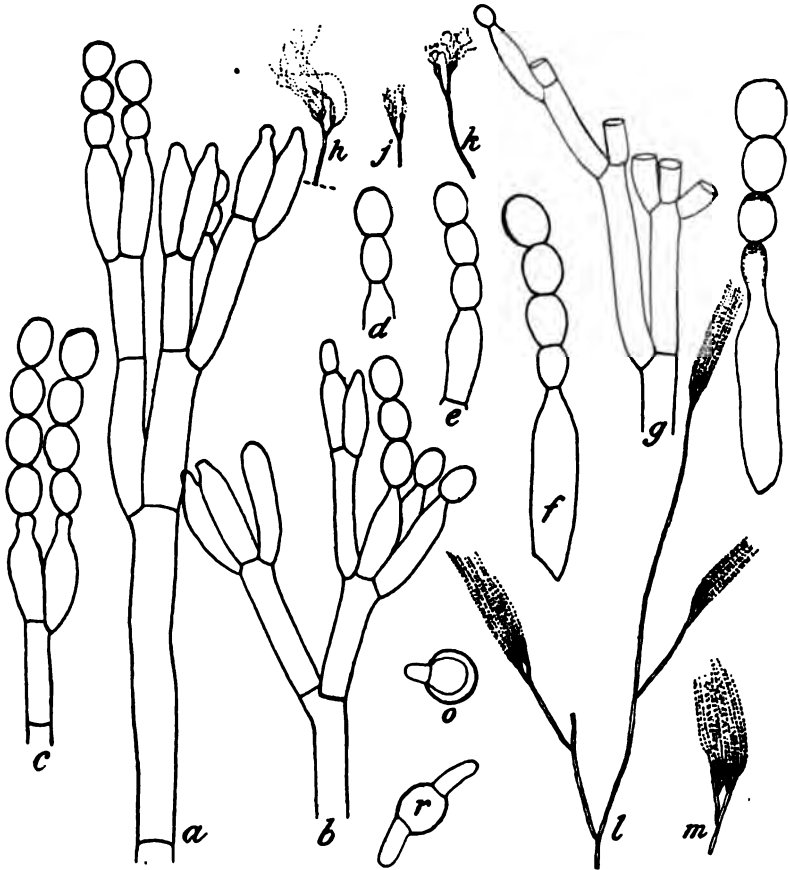


FIG. 18.—*Penicillium biforme*: a, b, g, branching of conidial fructification from potato-agar culture ($\times 1,400$); c, d, e, f, conidiferous cells and conidia ($\times 1,400$); h, j, k, sketches of conidial fructifications on potato agar ($\times 140$); l, m, sketches of conidial fructifications on sugar gelatin ($\times 140$); o, r, germination of conidia ($\times 900$).

Fifteen per cent gelatin in water, typical floccose colony; liquefaction, partial in old acidified cultures, none in 15 days; litmus reaction, strongly alkaline (in nearly all media). Potato agar and bean agar, colonies consisting only of very short conidiophores green to drab in color, little or no floccose mycelium. Potato plugs, typical. Cohn's solution, slow, half normal growth, with odor.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, typical up to 50 per cent sugar, with acid reaction. Lactose 3 per cent, rich growth, typical. Lactic acid 0.9

per cent, typical, alkaline reaction when old, crystal drops. Levulose 3 per cent, typical. Galactose 3 per cent, slow development, typical. Glycerin 3 per cent, typical. Potato starch 3 per cent, typical colony, crystal transpiration drops. Butter-fat, typical colony, mycelial mass tinged violet.

Milk, rich growth; curdling (0.25 per cent calcium chlorid added), in 8 days; digestion, rather slow; color in milk, none.

At 37° C., no growth, slowly green when cooled; check at 20° C., typical.

***PENICILLIUM COMMUNE* n. sp.**

In examining numerous Petri-dish cultures made for the examination of milk by the bacteriologists of the Storrs Experiment Station, large numbers of colonies of *Penicillium* have been studied. A very large percentage of these colonies have a series of common characters which are constant enough to mark out a species, or, perhaps better, a group of races, between which differences are either minute or so complicated by the occurrence of other races with overlapping characters as to make their separation a matter of considerable doubt. One of these has been selected as the basis of the following diagnosis. This form is morphologically closely similar to *P. expansum* (see figs. 1 and 19). It, however, lacks entirely the ability to form coremia and fails to discolor the substrata, but grows well in certain culture solutions which markedly restrain *P. expansum*, of which it might possibly be regarded as a variety. From its abundance in the situations studied it has been designated as *P. commune*.

Latin diagnosis.—Coloniis in gelatina vel agar Solani tuberosi aut phaseoli cultis, viridibus, demum brunneolis, in substrato late crescentibus, zonatis; marginis crescentis parte aëria ex conidiophoris, centri atque ex hyphis plus minusve floccosis composita; reverso et substrato incolorato; conidiophoris plerumque 300 μ raro usque 700 μ longis; fructibus conicis 100–200 μ longis, cum ramis alternatis et verticillatis confertis, basidiis 8–9 $\times$ 3 μ cum apicibus brevibus acutis, catenas conidiorum longas parallelis gerentibus; conidiis primum cylindricis vel ellipticis, demum globosis, 3–4 μ diam., 5–6 μ incrassatis germinantibus, levibus, viridibus, in catenis manentibus submersis; coloniis in gelatina in parte lente liquefacientibus; odore mucidus.

Habitat in lacte, caseo, etc., Storrs, Conn.

Colonies in gelatin or in potato or bean agar, dull green, becoming brown when old, broadly spreading, zonate, with broad white growing margin composed only of conidiophores, in the older parts becoming floccose masses of interwoven hyphæ; reverse of colony and substratum never colored; conidiophores commonly 300 μ or less in length, sometimes up to 700 μ ; conidial fructifications commonly 100–200 μ in length, compact at base and broadening above, variously branching with branches appressed, and verticils of conidiiferous cells 8–9 by 3 μ , abruptly narrowed to produce conidia; conidia cylindrical to elliptical and finally globose 3–4 μ , becoming 4–5 μ or larger in germinating, smooth, green, persisting in chains in fluid mounts; colonies liquefy gelatin slowly or partially, softening rather than producing clear liquid, alkaline in media without sugar but acid with either cane sugar or lactose, having a strong "moldy" odor.

Habitat, common in food, dairy products, etc., Storrs, Conn.

CULTURAL DATA.

Color green, becoming brown when old; reverse cream or white; color in media, none.

Odor, strong, "moldy."

Fifteen per cent gelatin in water, good growth; liquefaction, none in 15 days, slow or partial in older cultures; litmus reaction, slowly alkaline. Potato agar and bean agar, characteristic. Potato plugs, characteristic. Raulin's fluid, characteristic, with odor "moldy." Cohn's solution, small colonies, trace of pink below.

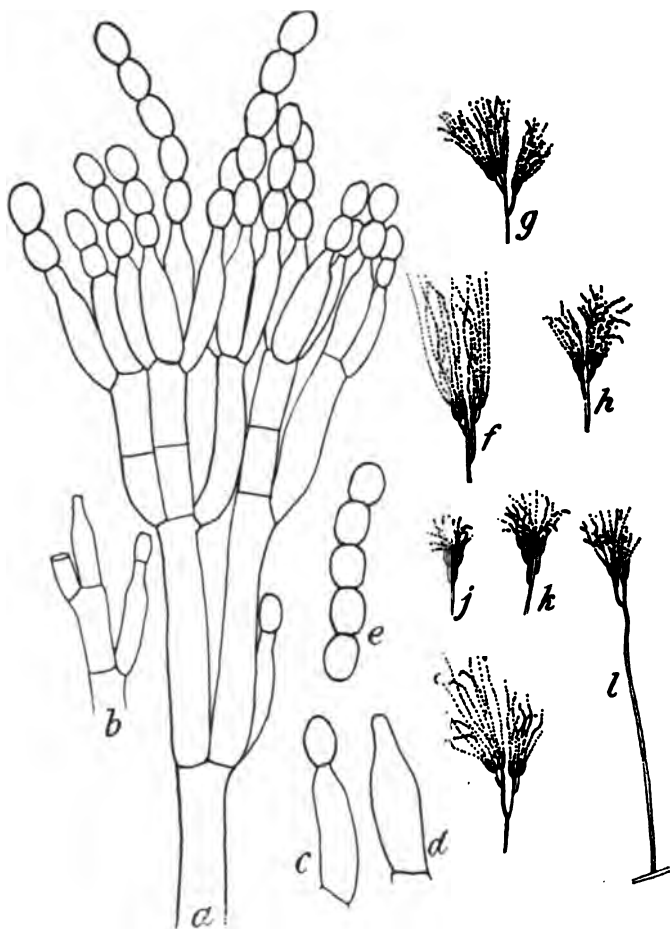


FIG. 19.—*Penicillium commune*: a, b, c, d, e, conidial fructification, branching, and production of conidia ($\times 900$); f, g, h, i, j, k, l, sketches of fructifications in various stages ($\times 140$).

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, typical culture, transpired drops crystal. Lactose 3 per cent, typical culture. Lactic acid 0.9 per cent, good growth. Levulose 3 per cent, good culture, strong odor. Galactose 3 per cent, good culture, strong odor, alkaline reaction. Glycerin 3 per cent, typical, strong odor. Butter fat, typical growth.

Milk, good typical colony; curdling (0.25 per cent calcium chlorid added) in 1 week; digestion, slow; color in milk, none.

At 37° C., no growth, grew upon cooling; check at 20° C., rich growth.

PENICILLIUM No. 22.

Colonies in gelatin or agar gray-green or glaucous persistently, or becoming gray, not brown, otherwise appearing as *P. commune*; conidial fructifications more loosely branching, with branches divergent; conidia larger and lighter color; odor, none or indefinite; reactions as in *P. commune*.

Habitat: Isolated several times from domestic soft cheeses made in the State of New York, 1904-5; found associated with *P. camemberti*; in appearance and color resembling latter species, but in structure of colony, measurements of conidiophores, etc., resembling *P. commune*. In pure culture this form has maintained its identity clearly for four years.

CULTURAL DATA.

Color gray-green; reverse colorless; color in media, none.

Odor, none or very slight.

Fifteen per cent gelatin in water, good growth; liquefaction, none in 15 days, partial in 2 to 3 weeks or more; litmus reaction, blue. Potato agar and bean agar, good typical colonies, with alkaline reaction to litmus. Potato plugs, good growth, typical gray-green colony with crystal drops of exuded water. Cohn's solution, medium growth, trace of pink in media.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, good growth up to concentration of 60 per cent, acid reaction. Lactose 3-10 per cent, typical. Levulose 3 per cent, good growth. Galactose 3 per cent, good growth, typical. Glycerin 3 per cent, good growth. Butter fat, rich growth, typical.

Milk, typical colonies; curdling (0.25 per cent calcium chlorid added) in 8 days; digestion, rather slow; color in milk, none.

At 37° C., no growth, grew when cooled; check at 20° C., typical.

PENICILLIUM CHRYSOGENUM n. sp.

Latin diagnosis.—Coloniis in gelatina vel agar Solani tuberosi aut phaseoli cultis, griseo-viridibus, demum brunneolis, in substrato late crescentibus, margine sterili lata juvenilibus parte aëria ex conidiophoris et caespitibus sparsis hyphorum adscendentium composita; reverso incolorato; conidiophoris plerumque singulatim usque $300 \times 4\mu$ orientibus, raro brevibus ex hyphis assurgentibus ramosis; fructibus conidicis 100-200 longis cum 1-2 ramis alternatis et divergentibus ramulos 1-2 verticillatos gerentibus; basidiis $8 \times 2.5\mu$ verticillatis ex apicibus ramulorum, catenas divergentes conidiiorum gerentibus; conidiis primum cylindricis vel ellipticis, demum globosis, 3-4 diam., pallide glaucis, magnis vacuolis; coloniis gelatinam liquefacientibus, alkalinis lacte; lactem, panem, gelatinam, auream colorantibus.

Habitat, in caseo, pane, etc., commune.

Cultivated in gelatin, or bean or potato agar, gray-green, becoming brownish when old; aerial portion consisting of conidiophores with some tufts of trailing aerial hyphæ; broadly spreading in the substratum with a wide sterile margin when young. Reverse of colony not discolored. Conidiophores mostly arising separately, up to 300 by 4μ , partly arising as short branches of aerial hyphæ; conidial fructifications 100-200 μ in length, with 1-2 alternate divergent branches, bearing alternate, verticillate or twice verticillate branchlets. Conidiiferous cells 8 by 2.5μ verticillate at the ends of branchlets bearing divergent chains of conidia. Conidia cylindrical or elliptical at first, then globose, 3-4 μ in diameter, pale green, with large vacuoles. Colonies liquefy gelatin, with alkaline reaction to litmus and produce in milk, bread, gelatin, and other substances a golden yellow color (from which the name).

Habitat, in bread, cheese, etc., apparently common and appearing in several varieties which differ in the intensity of color production, in appearance in certain cultures, but which are so far scarcely distinguishable by structural characters.

The culture here named *P. chrysogenum* has been kept under observation for more than four years without change. In this time several forms have been collected or sent to this laboratory which also produce the golden color in the digestion of milk suggestive of the name proposed. The cultural characters of these races differ in some degree in several cases, in others substantial identity has been observed. In reporting cultural data three numbers are included for comparison with this, viz, Nos. 25, 35, and 44. These agree in the following characters: Spreading habit, surface mostly

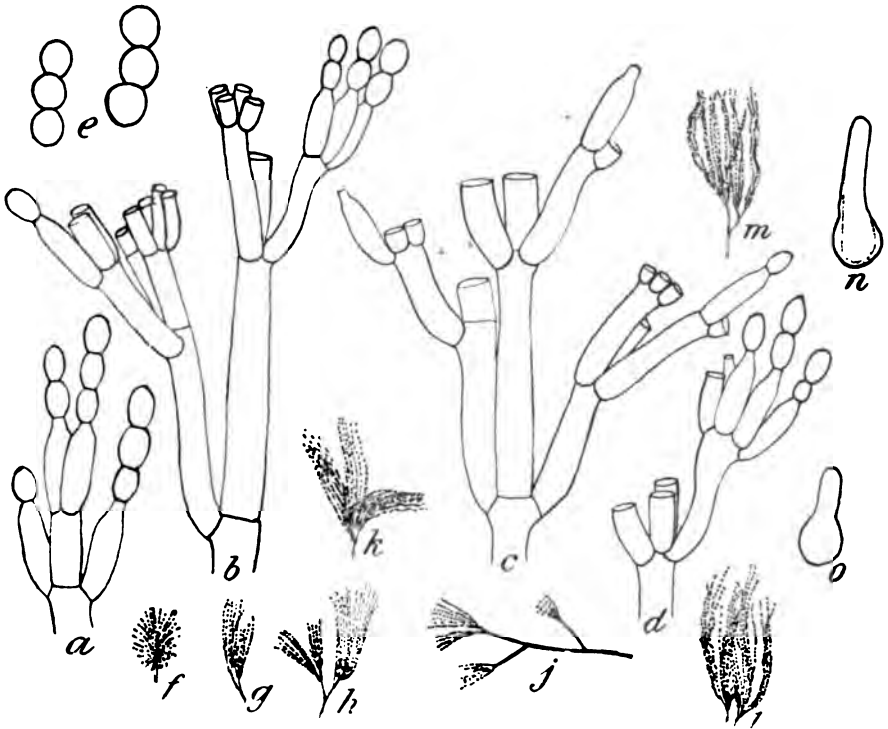


FIG. 20.—*Penicillium chrysogenum*: 'a, b, c, d, e, branching of conidial fructification from gelatin plates ($\times 900$); f, g, h, j, l, m, sketches of conidial fructifications from potato-agar plates ($\times 140$); n, o, germination of conidia ($\times 900$).

of conidiophores averaging about 300μ in length, nearly the same shade of green in color, similar morphology of conidial fructification, digestion of milk, gelatin, etc., with the production of yellow or golden color, liquefaction of gelatin progressive but slower than the expansion of the colony in the substratum so that a growing border of submerged vegetative hyphæ extends into hard gelatin for considerable time, in contrast to forms in which the colony becomes a floating "island" in a pool of fluid within the first week. In spite of these common characters colonies of these four races often show

great apparent differences in parallel culture in some specialized media. It seems preferable to let this name stand in a broad enough sense to include the forms having the common characters above noted than to attempt any narrower delimitation of this group of forms at this time.

Serial No. 57.—Another form which may be included temporarily with this group does not produce a yellow color in the substratum at all, but produces mycelium of orange color as seen from below. The surface growth is clear green without definite differences from the *chrysogenum* group, but the orange color in reverse remains as a constant difference from that group, unattended by any coloration of the medium.

CULTURAL DATA.

(Cf. Nos. 25, 35, and 44 in tables.)

Color gray-green, green, to brown when old; reverse colorless, or slight yellowish. Color in media golden yellow in certain media, no color in others.

Odor, none.

Fifteen per cent gelatin in water, good growth, yellow color in gelatin; liquefaction rapid—6–10 days, more or less rapid and complete in all gelatin media used; litmus reaction, alkaline. Potato agar and bean agar, good colonies, but no yellow color. Potato plugs, typical, potato becoming yellow. Raulin's fluid, typical with yellow transpired drops, but no yellow in fluid. Cohn's solution, growth, but not normal.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, good growth up to 30 per cent, reaction acid, fluid colorless. Lactose 3 per cent, fair growth, not heavy, fluid yellow, with acid reaction. Lactic acid 0.9 per cent, some growth. Galactose 3 per cent, good growth. Glycerin 3 per cent, colony white to green, no yellow color in fluid. Potato starch 3 per cent, good growth, yellow drops above, yellow in fluid. Butter fat, rich growth, with fluid golden yellow.

Milk, rich growth; curdling (0.25 per cent calcium chlorid added) in 1 week; digestion rapid; color yellow.

PENICILLIUM RUGULOSUM n. sp.

Latin diagnosis.—Coloniis in gelatina vel agaro phaseoli cultis, flavo-viridibus, dein viridibus, demum atro-viridibus, late crescentibus in agaro; parte aëria ex conidiophoris creberrimis et hyphis aëreis et paucis composita; reverso luteo et in parte aurantiaco imprimis in tubere Solani; conidiophoris 100–200×2.5–3 μ , singulatim vel ex hyphis aëreis prope substratum orientibus; fructibus conidiciis 100–150 μ longis (in saccharo multo longioribus) ex ramis 10–15×2.5 μ , compacte verticillatis, verticillos basidiorum, vel ramulorum, vel ramulorum et basidiorum eodem verticillo gerentibus; basidiis 9–12×2 μ , acuminatis, catenas longas et divergentes conidiorum gerentibus; conidiis 3.4–3.8×2.5–3 μ , ellipticis, viridibus, uno apice incrassato, verruculosus maturis, in catenis manentibus submersis, 5 μ diam. incrassatis gerinantibus; coloniis non (vel solum in parte et lente) gelatinam liquefacientibus.

Commune in culturis, Storrs, Conn.

Cultivated in gelatin or bean agar, yellowish green, then green, at length dark green; surface growth of densely crowded conidiophores with few aerial hyphæ interspersed at their bases; reverse of colonies yellow to orange in spots, especially upon potato or upon sugar media; substratum not or slightly yellowed; conidiophores 100–200 by 2.5–3 μ , arising separately or branching from aerial hyphæ just above the substratum; conidial fructifications 100–150 μ in length, consisting of appressed, verticillate branches

10–15 by 2.5μ , bearing verticils of conidiiferous cells, of branchlets, or of conidiiferous cells and branchlets together; conidiiferous cells 9–12 by 2μ , acuminate, bearing long divergent chains of conidia; conidia 3.4–3.8 by $2.5\text{--}3\mu$, elliptical, green, mostly with swelling at one end, verruculose when ripe, swelling to 5μ and germinating by one or two tubes; colonies do not or only partially liquefy gelatin.

Common in cultures, Storrs, Conn. Characterized by its verrucose spores and the brilliant color of the mycelium viewed from below.

CULTURAL DATA.

Color green or yellowish green; reverse yellow to orange or reddish orange; color in media, none.

Fifteen per cent gelatin in water, slow development; liquefaction, none or slight in old colonies; litmus reaction neutral. Potato agar, good growth, alkaline. Bean agar + 5 per cent cane sugar, rich heavy growth producing conidia in heavy dark-green masses, easily broken off by shaking the tube. Potato plugs, green, reverse bright orange.

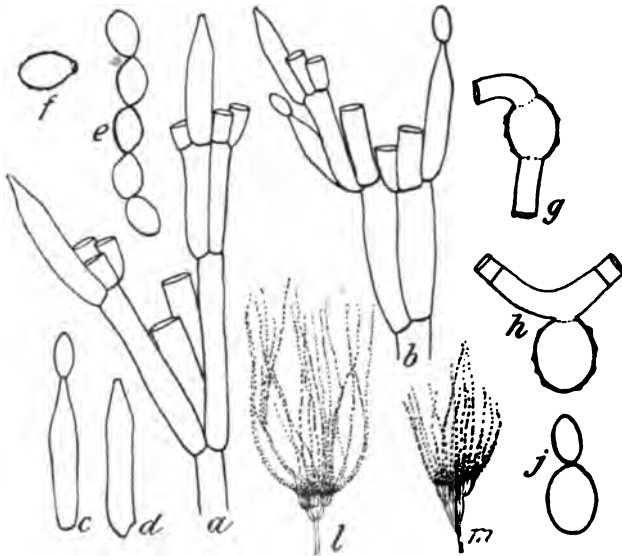


FIG. 21.—*Penicillium rugulosum*. a, b, branching of conidiophore ($\times 1,600$); c, d, e, conidiiferous cells (basidia) and conidia ($\times 1,600$); f, fully ripe conidium, showing delicate roughening of walls ($\times 1,600$); g, h, j, swelling and germination of conidia ($\times 1,600$); i, m, diagrams of conidial fructifications ($\times 260$).

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, growth in concentration up to 60 per cent, acid. Lactose, 10 per cent, slight growth. Levulose, fair growth, acid reaction; galactose, good growth; glycerin, slow growth; butterfat, slowly typical colony, yellow below; fluid, slightly yellowish.

Milk, growth typical, reverse yellow to orange or reddish orange; curdling (0.25 per cent calcium chlorid added) in 9 days; digestion slow or slight; color, none in milk.

At 37°C ., no growth, grew when cooled; check at 20°C ., typical.

PENICILLIUM CITRINUM n. sp.

Latin diagnosis.—Coloniis in gelatina vel agar Solani tuberosi aut phaseoli cultis, aeruginoso-viridibus, demum fulgineis; fructibus viridibus usque ad marginem gestis, i. e. margine sterile angustissima; coloniis in gelatina rotundis, parvis, cito liquefa-

centibus; in agaro latoribus; parte aëria ex conidiophoris et fructibus conidicis creberrimis composita, interdum caespitibus paucis hyphorum adscendentium in medio; reverso incolorato; conidiophoris (sine ramis) non longioribus 150μ , singulatim orientibus, aut paucis ex hyphis adscendentibus ramosis; fructibus conidicis 3-5 ramorum $16-30 \times 3\mu$, apice 5μ incrassatorum, in verticillo, basidia in verticillis compactis gerentum; utroque verticillo catenis conidiorum in columno compacto $50-150\mu$ longos adhaerentibus; basidiis $6-7 \times 2-3\mu$; conidiis globosis, $2.4-3\mu$ raro 3.5μ diam., aeruginoso-glaucis, granulatis intus, in catenis manentibus submersis.

Coloniis, saccharo commixto, substrata citrina in colore efficientibus (ubi nomen).

Habitat, in caseo, pane, etc., commune (?).

Colonies grown upon gelatin and potato or bean agar blue-green when young, becoming dark brown when old, with colored fruit borne almost to the very margin

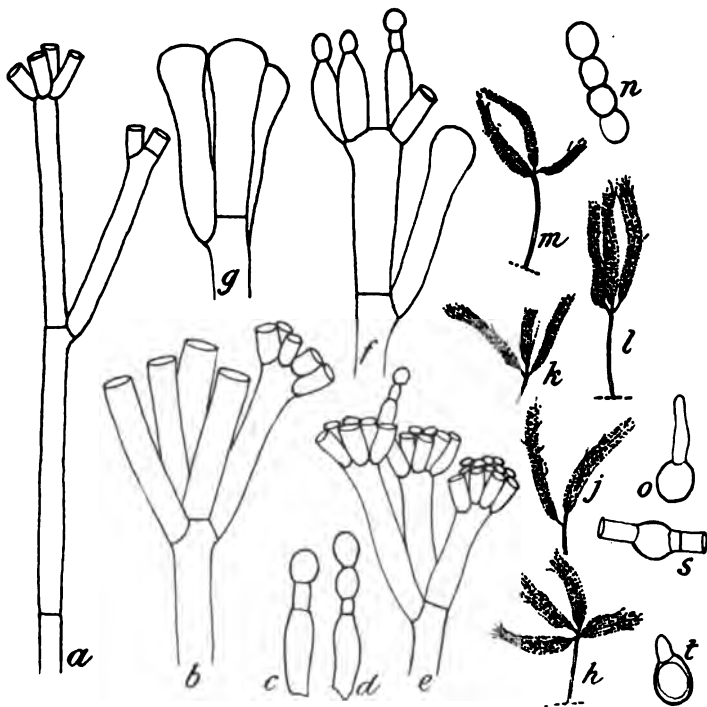


FIG. 22.—*Penicillium citrinum*: a, b, e, f, g, branching of conidial fructification, showing number of branches in each verticil and enlargement of ends of branches (a $\times 900$, others $\times 1,600$); c, d, n, conidiiferous cells and the formation of conidia ($\times 1,600$); h, j, k, l, m, sketches of conidial fructifications ($\times 140$); o, s, t, germination of spores ($\times 900$).

of the colony, so that the white border of submerged mycelium and uncolored fruit is very narrow; restricted in growth to a few millimeters in diameter upon gelatin, but becoming much larger upon agar; aerial part of colony consisting of densely standing conidiophores and conidia except in the center, where there arise a few tufts of trailing aerial hyphæ. Reverse of colony itself colorless or only yellowish. Conidiophores arising separately, rarely longer than 150μ , branching acropetally from submerged hyphæ radiating from the center of the colony, or branched from the hyphæ of the central aerial tuft. Conidial fructification a verticil 2 to 5 branches $16-30$ by 3μ enlarged at apex to 5μ , each producing a compact verticil of conidiiferous cells bearing chains of conidia massed together into columns $50-150\mu$ in length (usually $80-100\mu$).

The whole fructification appears in this way double, triple, or quadruple or even more complex by a secondary verticil from the central branch. Conidiiferous cells 6-7 by 2-3 μ . Conidia globose when ripe, 2.4-3 μ (even 3.5 μ diam. in cane-sugar cultures) in diameter, bluish-green, slightly granular in contents, adhering in chains in fluid mounts, losing vitality rapidly with change of color in old colonies. Colonies liquefy gelatin rapidly, so that they lie in pools of liquid within a week. Litmus reaction in plain gelatin, strongly alkaline. Produces a lemon-yellow color soluble in alcohol in media containing sugars, milk, gelatin, bread, and potato.

Found in cultures from milk and cheese, probably cosmopolitan.

Could this be *P. citreo-nigrum* Dierckx?

CULTURAL DATA.

Color bluish green, becoming dark brown when old if exposed to light; reverse colorless or yellowish; color in media lemon-yellow when cane sugar or gelatin or peptone is present, none in some media.

Odor, none.

Fifteen per cent gelatin in water, characteristic growth, becoming white by secondary sterile mycelium; liquefaction rapid, colonies floating in 5-6 days; litmus reaction alkaline. Potato agar and bean agar, colonies spreading more than upon gelatin, agar not colored or slightly colored. Potato plugs, typical growth, potato colored yellow, with yellow drops transpired; Raulin's fluid, typical growth, very slight yellow color. Cohn's solution, good growth, no yellow color.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, good growth with yellow color up to 30 per cent, with acid reaction. Lactose 3 per cent, slow poor growth giving a violet tinge instead of yellow color. Lactic acid 0.9 per cent, slight growth. Levulose 3 per cent, small characteristic growth. Galactose 3 per cent, good growth, reaction neutral or slightly acid. Glycerin 3 per cent, very small colonies, no color in medium. Alcohol, 5 per cent, good growth, no yellow. Potato starch 3 per cent, good growth, slight if any yellow. Tartaric acid, slow colorless colony. Butterfat, rich growth, lemon-yellow fluid.

Milk, rapid growth; curdling (0.25 per cent calcium chlorid added) in 10 days; digestion rapid; coloration pale yellowish.

At 37° C., slow growth, white colony, no color in medium; check at 20° C., rich growth, green, yellow in medium, in bean agar with cane sugar.

PENICILLIUM No. 37.

(Var. of *P. citrinum*? Or allied to *P. citrinum*?)

Colonies in media without sugar, green, gray-green, or gray; with sugar persistently green; surface velvety strict, composed of short crowded conidiophores up to 100 μ in length, branching from closely woven mycelium partly submerged, partly aerial, with margin narrow, not widely spreading in the substratum; reverse of colony and substratum not colored or creamy; conidial fructifications sometimes a single verticil of conidiiferous cells, sometimes 2 to 4 verticillate branches; chains of conidia from each verticil forming a column up to 500-600 μ in length in sugar media; branches of fructification 13-14 by 2-2.5 μ enlarged at apex; conidiiferous cells 8-10 by 2+ μ abruptly narrowed into sterigmata, usually 6-10 in each verticil; conidia broadly elliptical to globose, 2.5-3 μ at first becoming 4-5 μ before germinating, thin-walled, smooth, pale yellowish green, germinating by a single tube; colonies liquefy gelatin rapidly (6 to 7 days), with strong alkaline reaction to litmus.

Received from Prof. P. H. Rolfs, Miami, Fla., in culture upon bean stems, 1905.

Allied to *P. citrinum* by morphology and culture reactions, but differing in lacking the power to color media yellow and in its greater dependence for typical growth upon the presence of cane sugar.

CULTURAL DATA.

Color light blue-green, olive, or gray in various media; reverse, white or cream; color in media, none or slightly yellowish.

Odor, none.

Fifteen per cent gelatin in water, small olive-green colonies; liquefaction, rapid—6 to 7 days; litmus reaction, alkaline. Potato agar and bean agar, typical. Potato plugs, typical. Raulin's fluid, typical. Cohn's solution, slow and weak-growing colony.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, good growth even up to 60 per cent, acid reaction. Lactose, 3 per cent, slow abnormal growth. Lactic acid, 0.9 per cent, small but characteristic colony. Levulose, 3 per cent, small colo-

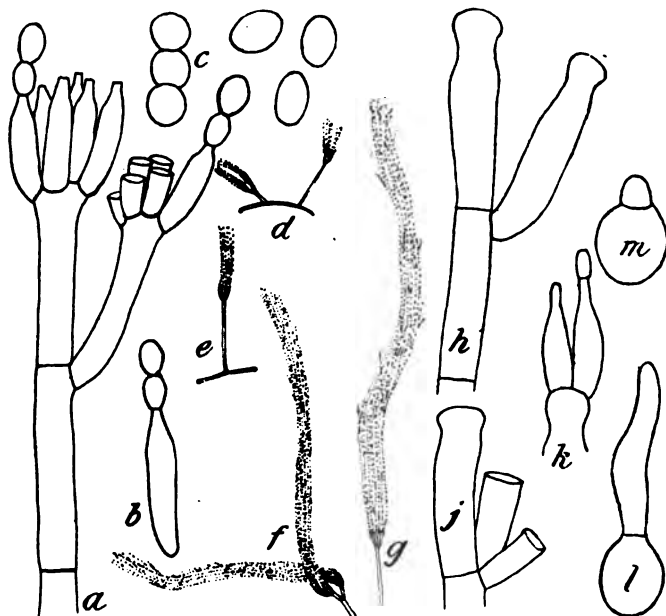


FIG. 23.—*Penicillium* No. 37: *a*, typical branched fructification, two verticils of conidiferous cells ($\times 1,600$); *b*, *c*, conidiferous cell and conidia ($\times 1,600$); *d*, *e*, sketches of conidial fructifications from potato-agar culture ($\times 140$); *f*, *g*, sketches from an old culture on 3 per cent cane-sugar agar, showing simple (*g*) and branched (*f*) forms ($\times 140$); *h*, *j*, *k*, branching of conidiphore, swollen ends of branches ($\times 1,600$); *l*, *m*, germination of conidia ($\times 1,600$).

nies. Galactose, 3 per cent, typical. Glycerin, 3 per cent, small growth. Potato starch, typical. Butterfat, slow growth, deep heavy green colony.

Milk, curdling (0.25 per cent calcium chlorid added), rapid; ^a digestion rapid and complete; color, none.

At 37° C., killed; check at 20° C., grew well.

PENICILLIUM No. 12.

This form differs from *P. citrinum* in producing no coloration of the medium and in producing conidial fructifications in which the chains of conidia are more or less divergent instead of aggregated into columns. In culture there is general corre-

^a The time of curdling is almost impossible to determine in cases where digestion begins quickly and progresses rapidly.

spondence in reactions, without identity; this form appears to be much more dependent upon cane sugar for the production of typical color of the conidia and growth than is *P. citrinum*. There is also a greater tendency to the production of a layer of mycelial hyphæ just above the surface of the substratum, from which the conidiophores arise as aerial branches.

This form was received from Prof. C. E. Marshall, Agricultural College, Michigan under the name of *P. glaucum*.

CULTURAL DATA.

Color pale blue-green; reverse of colony cream, not colored; color in media, none. Odor, none.

Fifteen per cent gelatin in water, rather small pale blue colonies, rapidly becoming white by secondary sterile growth of hyphæ; liquefaction, rapid—6 days; litmus reaction, alkaline. Potato agar, as in gelatin. Potato plugs, very poor growth, grayish or yellowish green. Raulin's fluid, slow but typical colonies, delicate blue; Cohn's solution, slow and restricted growth.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, grows well in concentrations up to 60 per cent. Lactose 3 per cent, small colonies lacking nourishment. Lactic acid 0.9 per cent, small colonies floating in fluid. Levulose 3 per cent, good growth, alkaline reaction. Galactose 3 per cent, good growth, alkaline reaction. Glycerin 3 per cent, slow-growing colonies, becoming gray-brown when old. Potato starch, good colonies. Butterfat, slow and ill-nourished growth.

Milk, rapid growth; curdling (0.25 per cent calcium chlorid added) in 7 days; digestion rapid and very complete; color in milk, none.

At 37° C., killed in 6 days; at 20° C., good growth.

PENICILLIUM ATRAMENTOSUM n. sp.

Latin diagnosis.—Coloniis in gelatina vel agar Solani tuberosi aut phaseoli cultis, viridibus, parte aëria plerumque ex conidiophoris singulatim orientibus, medio cum hyphis aëreis interspersis, margine albo ex hyphis fertilibus angusta; reverso incolorato vel parum ochraceo; substrato aut incolorato aut in substratis saccharinis et in lacte atrobrunneo tarde fere atro; conidiophoris 240–300 usque 400 μ longis; fructibus conidicis 100 usque 200 μ longis, ramis 1–2 verticillatis 2–4 inæqualiter longis in verticillo in apice incrassatis; basidiis 8–10 μ longis, parallelis in verticillo; catenis conidiorum eodem verticillo in columno compactis; conidiis ellipticis, 3.5–4 (usque 4.8) $\times$ 2.5–3 usque 3.5 μ , lævibus, viridibus, 6–7 μ incrassatis et uno tubo germinantibus; coloniis gelatinam cito liquefacientibus, alkalinis lacmo; odore in lacte proprio, in substratis aliis nullo.

Ex caseo cultum, Storrs, Conn., 1905.

Affine *P. citrino*.

Colonies upon gelatin or upon potato or bean agar bright green, aerial part mostly of simple conidiophores, mixed in older parts with branching aerial hyphæ but narrowly spreading at the margin by new conidiophores only. Reverse of colonies shows a slight production of yellow (ochraceous) color. Conidiophores 240–400 μ , averaging about 300 μ in length. Conidial fructification up to 200 μ in length, usually 100 μ or less, verticillately or twice verticillately branched; branches 2–4 in a verticil divergent, unequal in length, swollen at ends, bearing conidiiferous cells. The conidial chains from each verticil form a dense column, which diverges more or less from the other columns when old. Conidiiferous cells 8–10 μ in length, closely parallel. Conidia elliptical, varying from 3.5–4 μ by 2.5–3 μ on agar, somewhat larger in gelatin cultures, up to 4.8 by 3.5 μ , smooth, homogeneous green with a slight yellowish shade when seen in mass, swelling to 6–7 μ in diameter and germinating by a single tube. Mycelial cells 5–7 μ in diameter and up to 30 μ or more in length. Colonies liquefy sugar-gelatin

rapidly, give an alkaline reaction to litmus, digest milk, and color potato agar containing high percentage of sugar a deep black.

Found upon Camembert cheese imported from France. Closely related morphologically to *P. citrinum*, from which it is separated by the longer conidiophores and larger spores as well as the black discoloration of sugar media.

CULTURAL DATA.

Color deep (blue) green to brown when old; reverse uncolored, or brown in some media; color in media, none, or brownish to almost black.

Odor, none.

Fifteen per cent gelatin in water, deep green, brown when old, rich growth; liquefaction, 7 days, varies from 6 to 12 days in other gelatin media; litmus reaction, alkaline. Potato agar and bean agar, typical, no color below. Potato plugs, dark green, potato blackened. Raulin's fluid, rather weak growth. Cohn's solution, germinated only.

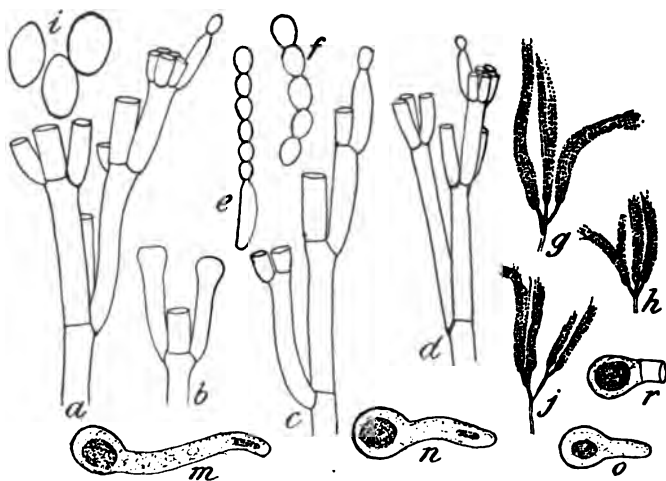


FIG. 24.—*Penicillium atramentosum*: a, b, c, d, branching of conidial fructification showing unequal length of branches, swollen ends ($\times 900$); e, f, conidiiferous cell and chain of conidia ($\times 900$); g, h, f, sketches of conidial fructifications ($\times 140$); i, conidia ($\times 1,600$); m, n, o, r, germination of conidia ($\times 900$).

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, good growth up to 50 per cent, with acid reaction in 50 per cent solution. Lactose 3 per cent, small and slow growth. Lactic acid 0.9 per cent, no growth. Levulose 3 per cent, slowly typical. Galactose 3 per cent, typical, with alkaline reaction. Glycerin 3 per cent, germination. Potato starch 3 per cent, good growth, no color in fluid or reverse of colony. Butterfat, typical, green colonies with reverse brown, and fluid uncolored.

Milk, curdling (0.25 per cent calcium chlorid added) in 9 days; digestion, rapid, fairly complete; color, brownish to almost black.

At 37° C., no growth, grew when cooled; check at 20° C., typical.

PENICILLIUM No. 24.

(Related to *P. atramentosum*?)

Colonies upon gelatin and potato or bean agar blue-green, becoming brown rapidly when old, or smoky with very dense velvety surface consisting of conidiophores arising in the substratum or just above its surface, with a very abrupt narrow white

margin of unripened fruit and submerged mycelium during the growing period. Reverse of colony and medium colorless under all conditions studied. Conidiophores from 100 to 400 μ , averaging about 250 μ , in length, either arising separately or as lateral branches of hyphae just above the substratum. Conidial fructification up to 200 μ in length produced by various branching from the conidiophores in which each primary branch is often divergent to produce separate mass of conidia. Conidiiferous cells 7-10 by 3 μ . Conidia globose 3.3-4 μ , homogeneous blue-green, smooth, seeming to lose vitality rapidly under laboratory conditions. Colonies liquefy gelatin in 7 to 12 days so that they lie in pools of liquid. Litmus reaction strongly alkaline.

Found in the cultures from Camembert cheese in the laboratories at Storrs, Conn. Differs from the preceding and from *P. citrinum* by its longer conidiophores, the alternate branching of its fructification, the size of its spores, and by failure to color the substrata. The relative value of ellipticity of conidia as a diagnostic character appears to be questionable. This form is therefore presented under cultural number

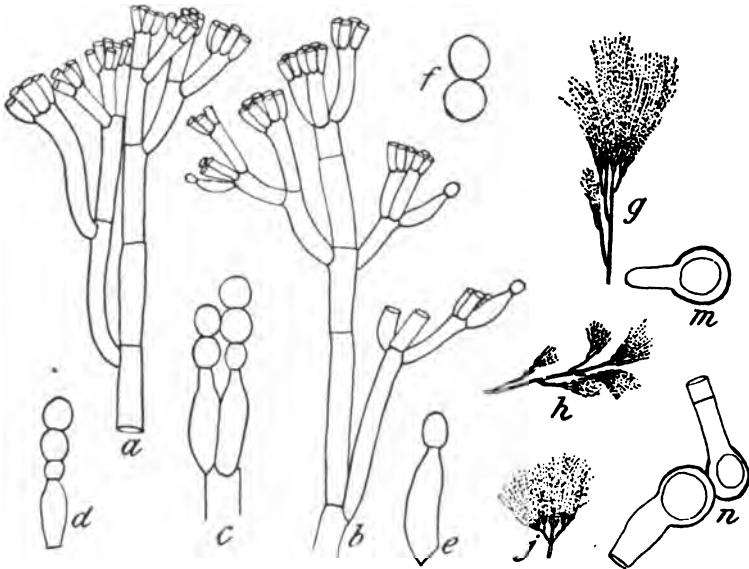


FIG. 25.—*Penicillium* No. 24: a, b, branching and arrangement of branches in conidial fructification ($\times 140$); c, d, e, conidiiferous cells and conidia ($\times 1,400$); g, h, j, sketches of form and arrangement of conidiophores ($\times 140$); m, n, germination of conidia ($\times 900$).

only, whereas the preceding has been identified from accidental cultures more frequently, and hence is given name and description as a species.

CULTURAL DATA.

Color deep green (blue-green), becoming brown when old and exposed; reverse white or cream; color in media, none.

Odor, none.

Fifteen per cent gelatin in water, characteristic colony; liquefaction, rapid—11 days or even less; litmus reaction alkaline. Potato plugs, deep blue-green, crystal drops. Raulin's fluid, weak but characteristic growth. Cohn's solution, slow but characteristic development.

Synthetic fluid (Dox's), carbon supplied: Cane sugar, good growth up to 30 per cent, acid reaction. Lactose, 3 per cent, slow development, not typical. Lactic acid 0.9 per cent, good colony. Levulose 3 per cent, small colonies. Galactose 3

per cent, good growth, strongly alkaline. Glycerin 3 per cent, weak growth. Potato starch, characteristic colony.

Milk, curdling (0.25 per cent calcium chlorid added) in 8 days; digestion medium rapid; color in milk, none or ?

At 37° C., no growth, grew when cooled; check at 20° C., good typical.

***PENICILLIUM STOLONIFERUM* n. sp.**

Latin diagnosis.—Coloniis in gelatina vel agar Solani tuberosi cultis, viridibus vel flavo-viridibus, demum griseo-viridibus vel griseis in agar sine saccharo, cum sac-

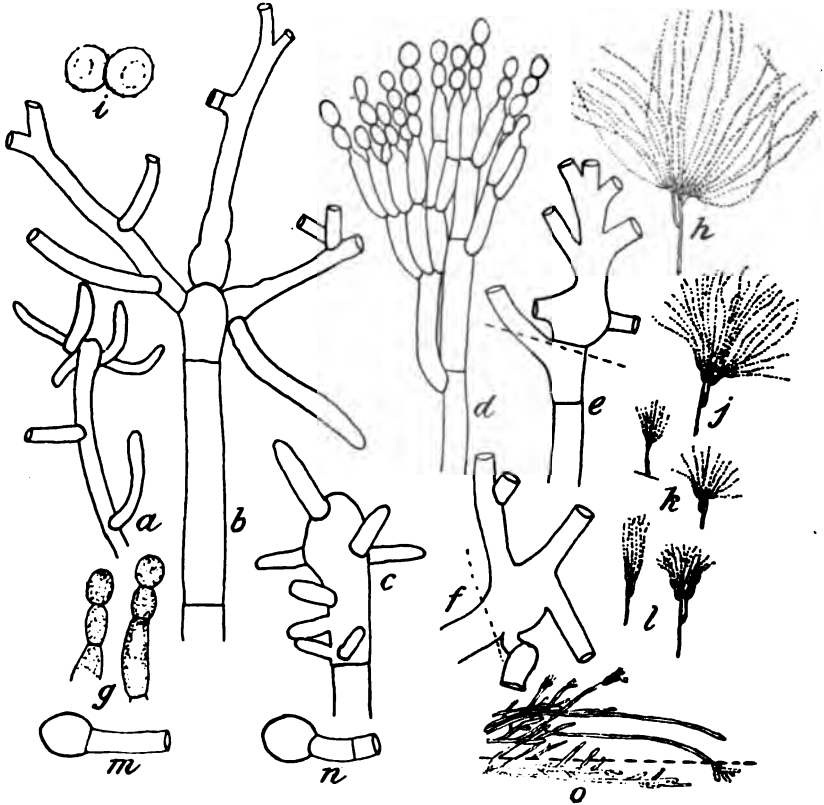


FIG. 26.—*Penicillium stoloniferum*: a, b, c, e, f, the types of branching at the tips of the "stolons" by which this species spreads in substrata (b, c, e, f. $\times 900$); d, conidial fructification ($\times 900$); h, j, k, l, sketches of conidial fructifications of various ages, h and j being characteristic shapes ($\times 140$); g, formation of conidia ($\times 900$); i, ripe conidia showing minute granulation ($\times 1,600$); m, n, germination of conidia ($\times 900$); o, rough diagram of habit.

charo viridibus, floccosis, in culturis juvenalibus stolonibus aereis citius quam hyphis submersis crescentibus, reverso incolorato vel in parte flavo; conidiophoris brevibus ex hyphis adscendentibus ramosis, usque 100μ longis, aut singulatim orientibus (sine ramis) plus minus 300μ longis; fructibus conidicis $40-80\mu$ raro usque 170μ longis, ex ramis brevibus compactis, et basidiis verticillatis, in baside confertissimis, catenas conidiorum late divergentes gerentibus compositis, interdum ramus infimus tam divergens ut fructus duplex videatur; basidiis $10 \times 3\mu$; conidiis ellipticis vel paene globosis, $2.8-3.4\mu$ diam., pallido flavo-viridibus laevibusque; coloniis gelatinam cito liquefacientibus, alkalinis lacmo.

Habitat, in fungis putrescentibus, Boletis, Polyporis; Storrs, Conn.; Paris, Gallia.

Cultivated in gelatin or potato agar, green or yellowish green, becoming gray-green or gray when old (remaining green in sugar media), floccose, spreading more rapidly in young cultures by aerial stolons than by submerged hyphæ (i. e., the submerged mycelium seems to arise from the aerial rather than vice versa); reverse of colony not colored or partly yellow; conidiophores arising as short branches (100μ or less in length) from aerial hyphæ, or arising separately 300μ or more in length especially at the margins of older colonies; conidial fructification 40–80 more rarely up to 170μ in length, composed of short appressed branches and numerous conidiiferous cells densely crowded at the base bearing very loosely divergent chains of conidia; sometimes the lowest branch diverges so that the fructification appears double; conidiiferous cells 10 by 3μ ; conidia slightly elliptical or globose, 2.8– 3.4μ , smooth, yellowish green in mass, almost hyaline by transmitted light; colonies liquefy gelatin very rapidly, with a strong alkaline reaction to litmus.

Habitat, decaying fungi, Boleti, Polypori; cultures from milk and ensilage. Collected repeatedly at Storrs, Conn.; once upon decaying *Boletus scaber* at the Jardin des Plantes in Paris, hence probably widely distributed. The stolon-producing character is so easily seen and so characteristic of this species as to seem adequate to distinguish it from all other species studied. This has been observed upon a decaying *Boletus* with a hand lens.

CULTURAL DATA.

Color white to yellowish green, deep green becoming yellowish brown or gray in old cultures; reverse, not colored (or slightly yellow); color in media, none or slight. Odor, none.

Fifteen per cent gelatin in water, good growth, yellowish green; liquefaction, rapid in all gelatin media; litmus reaction, strongly alkaline. Potato agar, good growth, pale green to gray. Bean agar, good growth, pale green to gray. Potato plugs, good growth, deep green, transpired drops brown. Raulin's fluid, slow but characteristic colonies. Cohn's solution, typical growth.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, good growth up to 50 per cent, conidial areas persistently green (viridis to atro-viridis), acid reaction. Lactose 3 per cent, slow abnormal colonies, weak. Lactic acid 0.9 per cent, small colonies becoming alkaline. Levulose 3 per cent, typical colonies, alkaline reaction. Galactose 3 per cent, typical, alkaline reaction. Glycerin 3 per cent, good growth, not heavy. Potato starch 3 per cent, typical, drops yellow, fluid colorless.

Milk, typical colonies; curdling (0.25 per cent calcium chlorid added) in 1 week; digestion, rapid; color in milk, little or none.

At 37°C ., killed; check at 20°C ., good colony.

PENICILLIUM FUNICULOSUM n. sp.

Latin diagnosis.—Coloniis in gelatina vel agaro Solani tuberosi aut phaseoli cultis, atro-viridibus, late crescentibus, floccosis; parte aëria ex hyphis decumbentibus, ramosis, cæspitosis, late intricatis, et fasciculatis, conidiophoros breves gerentibus interdum hyphos secundarias albas floccosas lente evolvente; reverso rubescente demum atro-vinoso; substrato-aut lacte-aut gelatina, vinoso; conidiophoris (sine ramis) 20–80 usque 100μ longis, plerumque ex hyphis repentibus vel fasciculatis, interdum singulatim orientibus, fructibus conidicis usque 125– 160μ longis, cum 1, 2 ramis alternatis, dein ramulis verticillatis, basidia in verticillos densos catenis conidiorum parallelis gerentibus; basidiis 10–14 $\times$ 2– 3μ , parallelis in verticillo, acuminatis; conidiis primum cylindricis, demum fusiformibus vel ellipticis, 3–4 $\times$ 2– 3μ , viridibus; conidiorum catenis solventibus submersis; coloniis gelatinam non liquefacientibus, acidis lacmo, siccantibus senescentibusque interdum coremiis paucis evolventibus.

In cultura, Storrs, Conn., 1905; communicavit Dr. E. A. Bessey, Miami, Fla., 1908.

Cultivated in gelatin or potato or bean agar, deep green, broadly spreading, surface closely floccose with procumbent hyphæ, tufts and ropes of hyphæ bearing lateral conidiophores; reverse becoming red, purple, or very dark purple, almost black, with the whole mass of medium colored; conidiophores short, 20–80 or 100 μ , mostly perpendicular branches from trailing hyphæ, sometimes arising separately from the substratum; conidial fructification up to 125 or 160 μ in length, with 1 or 2 alternate appressed branches bearing verticillate branchlets and dense verticils of parallel conidiiferous cells 10–14 by 2–3 μ ; conidia at first cylindrical, then elliptical or fusiform, 3–4 by 2–3 μ , green, in chains which break up completely in fluid mounts; colonies not liquefying gelatin in 2 weeks, with acid reaction to litmus.

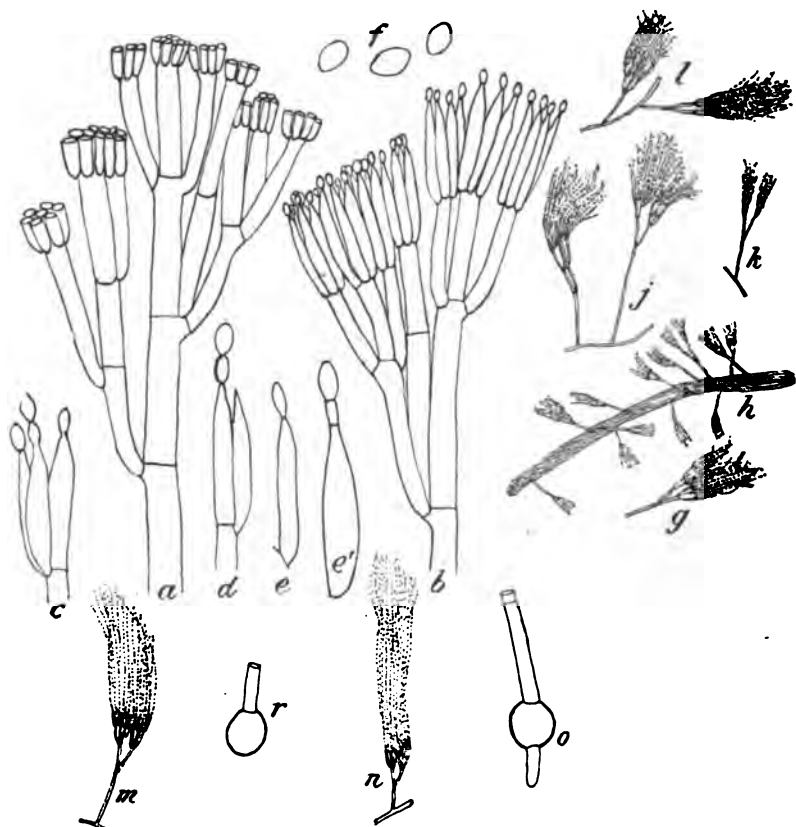


FIG. 27.—*Penicillium funiculosum*: a, b, c, d, e, f, conidial fructifications with conidiiferous cells and conidia ($\times 900$, except c, 1,600); g, h, j, k, l, m, n, sketches of fructifications, separate and borne upon hyphæ and ropes of hyphæ ($\times 140$); o, r, germination of conidia ($\times 900$).

Found in accidental culture, Storrs, Conn., 1905; also received from Dr. E. A. Bessey, Miami, Fla., 1908. Easily recognized in culture.

CULTURAL DATA.

Color, deep green with secondary floccose masses of mycelium in some cultures; reverse and color in media, red to very dark red, or, colorless in certain media.

Odor, none.

Fifteen per cent gelatin in water, thin, widespread but characteristic growth; liquefaction, none or very slight; litmus reaction acid. Potato agar and bean agar, typical

colonies with red color in medium. Potato plugs, typical, reverse of colony and potato both deep red. Raulin's fluid, good growth, but no color in fluid. Cohn's solution, germination only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar 3 per cent, good growth, but no red color. Lactose 3 per cent, very little growth. Lactic acid 0.9 per cent, little growth. Levulose 3 per cent, little growth. Galactose 3 per cent, little growth. Glycerin 3 per cent, germinated only, grew when sugar was added. Potato starch 3 per cent, good colonies, but no red color. Butterfat, rather small colonies with many delicate coremia, reverse of colonies red, with no color in fluid.

Milk, good growth with scattered coremia in old cultures; curdling (0.25 per cent calcium chlorid added) very slow—about 4 weeks; digestion slow or slight, no clear fluid; color in milk, colony deep red below, milk deep red (vinous) at top, shading to white below, very slowly colored.

Grew about equally well at 37° C. and 20° C.

PENICILLIUM DECUMBENS n. sp.

Latin diagnosis.—Coloniis in gelatina pura vel agar Solani tuberosi aut phaseoli cultis, griseo-glaucis, grisèis, demum brunneolis, sparsis; in saccharo officinaro commixto densior, glaucescentibus; parte aëria ex hyphis decumbentibus vel stoloniformibus conidiophoros brevissimos gerentibus, denuin cespitulis albis densis hypharum steriliuin secundariarum, conspersis; reverso incolorato; conidiophoris 20–100×3μ, basidiis 7–9×2–3μ, in uno verticillo denso gerentibus; fructibus conidiciis ex catenis conidiorum primum in columno usque 100μ longo, mox, in capitulo conglutinato solutis; conidiis globosis, 2.5–3μ, vacuolatis, lævibus, primum pallide glaucis demum brunneolis; coloniis gelatinam non liquefacientibus, alkalinis lacmo, saccharophilis, odorem in saccharo evolventibus.

Communicavit, Prof. P. H. Rolfs, Miami, Fla., 1905.

Cultivated in gelatin or potato agar, white to gray, gray-green ultimately yellowish brown, green in cultures with cane sugar, surface growth consisting of trailing or stolon-like hyphæ sparsely developed and so close to the substratum as to appear only as fertile hyphæ, bearing the conidiophores as short branches 20–100μ in length, in old colonies with dense tufts of sterile secondary mycelium scattered upon the surface; conidial fructifications consisting of single verticils of crowded conidiiferous cells, 7–9 by 2–3μ, bearing conidial chains first in loose columns up to 100μ in length but soon becoming enveloped and broken up in the drops of fluid secreted abundantly from the mycelium (*Gliocladium*-like); conidia globose 2.5–3μ, vacuolate, smooth, pale green then brownish in mass; colonies do not liquefy gelatin; give a weakly alkaline reaction to litmus; produce a definite odor in cultures containing cane sugar.

Contributed by Prof. P. H. Rolfs from Miami, Fla., 1905.

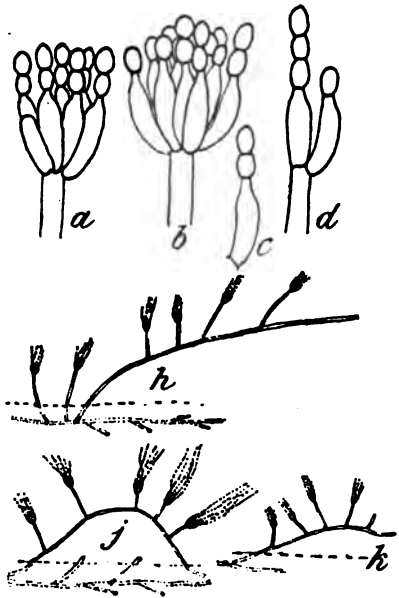


FIG. 28.—*Penicillium decumbens*: a, b, c, d, conidial fructification, a single verticil of conidiiferous cells (× 900); h, j, k, sketches of conidial fructifications, with diagram of habit and appearance of young culture on potato-agar (× 140).

CULTURAL DATA.

Color gray, gray-green, often gray or gray-brown when old; reverse white; color in media, none.

Odor, distinct in cane-sugar media.

Fifteen per cent gelatin in water, medium growth, gray-green to brown when old; liquefaction, none; litmus reaction neutral. Potato agar and bean agar, rather small colonies, weak growth, grayish green to yellow-brown. Potato plugs, white to yellowish brown colonies, very weak growth. Raulin's fluid, rich growth, bright green, distinct odor. Cohn's solution, germination only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, rich growth up to 30 per cent. Lactose 3 per cent, slight growth. Lactic acid 0.9 per cent, medium colony, light green. Levulose 3 per cent, small greenish colonies. Galactose 3 per cent, growth, faintly alkaline reaction. Glycerin 3 per cent, germination only. Potato starch 3 per cent, very slight growth. Butterfat, weak colonies.

Milk, not adapted to this species, colonies grow very slowly; curdling (0.25 per cent calcium chlorid added) slow—about 4 weeks; digestion, little or none; color in milk, none.

At 37° C. some growth; check at 20° C. better than 37° C.

***PENICILLIUM DIVARICATUM* n. sp.**

Latin diagnosis.—Coloniis in gelatina vel agar phaseoli cultis, avellaneis, nunquam viridibus, in substrato late crescentibus; parte aëria ex hyphis fertilibus intricatis, demum fere pulverulenta; reverso incolorato; hyphis fertilibus septatis, plerumque brevibus, repentibus vel adscendentibus; fructibus conidicis aut terminalibus aut lateralibus ex hyphis fertilibus repentibus ex verticillis sessiles ramorum et basidiorum, irregulariter in hyphis fertilibus orientibus; basidiis 15–20×3 μ , sterigmatibus longis acuminatis, in baside confertis, apice late divergentibus, catenas longas conidiorum gerentibus; conidiis ellipticis vel fusiformibus, 5–7×2.5–3 μ , avellaneis, 10 μ incrassatis 2–3 tubis germinantibus; coloniis gelatinam non liquefacientibus, alkalinis lacmo.

Legit, C. Thom, Storrs, Conn.

Cultivated in gelatin or bean agar, yellowish brown (avellaneous), never green, broadly spreading in the substratum; superficial growth consisting only of closely woven fertile hyphæ, becoming powdery in appearance when mature; reverse of colony not discolored; fertile hyphæ septate, usually short, mostly creeping; conidial fructifications either terminal or on short branches of creeping or partially erect hyphæ, consisting of separate conidiiferous cells, of verticils, or of series of verticils of branchlets and conidiiferous cells irregularly distributed along the fertile hyphæ; conidiiferous cells 15–20 by 3 μ , with long acuminate sterigmata, broadly divergent at the apices and bearing long chains of conidia; conidia elliptical or fusiform, 5–7 by 2.5–3 μ , yellowish to brownish, swelling in germination to 10 μ and producing 2 or more tubes; does not liquefy gelatin; litmus reaction alkaline.

Unmistakable when once seen in culture. Found in a mucilage bottle, Storrs, Conn., 1904. Later contributed by Prof. G. F. Atkinson from North Carolina.

In common with several other forms included in the genus *Penicillium*, the conidial fructifications of this species are not strictly penicillate and terminal. Every gradation is found from fruiting systems typical of the genus to simple chains of conidia borne by single cells or basidia upon prostrate or even submerged hyphæ. It partakes, however, of the cultural character of the species of the genus, as shown by its copious growth upon many different substrata.

CULTURAL DATA.

Color light clay to chocolate or yellow (avellaneous, nearly) to darker, approaching brownish yellow, never green; reverse uncolored; color in media, none.

Odor, none.

Fifteen per cent gelatin in water, good growth; liquefaction, none; litmus reaction, acid. Potato agar, bean agar, and potato plugs, typical. Raulin's fluid, typical. Cohn's solution, germination only.

Synthetic fluid (Dox's) carbon supplied as: Cane sugar, good growth up to 30 per cent, acid reaction. Lactose 3 per cent, slight growth. Lactic acid 0.9 per cent, medium growth, not fully normal. Levulose 3 per cent, small development. Galac-

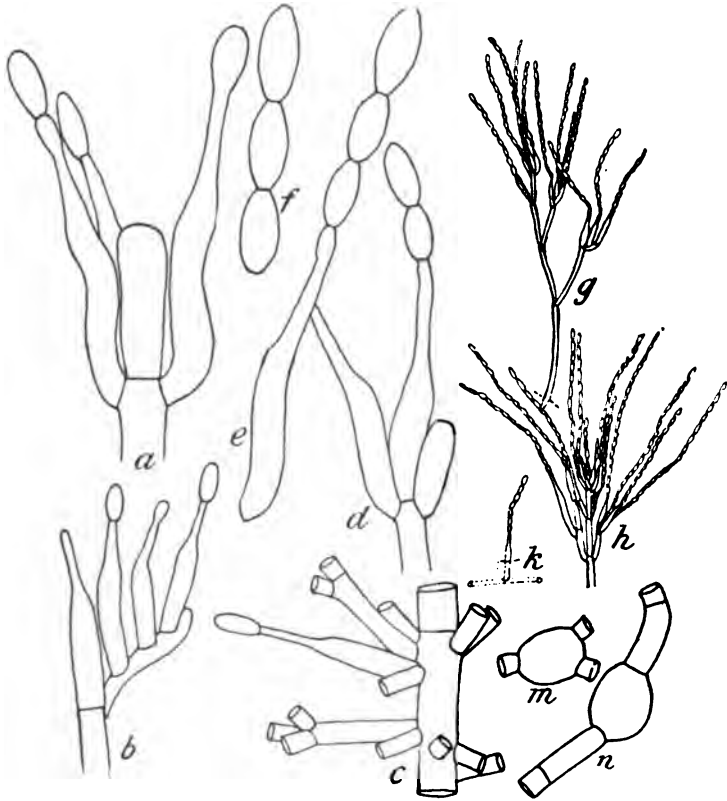


FIG. 29.—*Penicillium divaricatum*: a, d, e, f, conidiferous cells, conidia and their arrangement ($\times 1,600$); b, c, irregular types of arrangement; g, h, k, sketches of conidial fructification ($\times 260$); m, n, germination of conidia ($\times 900$).

tose 3 per cent, slow growth, reaction neutral. Glycerin 3 per cent, germinated only; grew when sugar was added. Potato starch 3 per cent, typical. Butter fat, weak growth, but characteristic fruiting.

Milk, slow and weak colonies; curdling (0.25 per cent calcium chlorid added) slow—14 days or more; digestion, slow and slight; color in milk, none.

Colonies grew better at 37° C. than at 20° C.

PENICILLIUM LILACINUM n. sp.

Latin diagnosis.—Coloniis in gelatina vel agar phaseoli cultis, albis, vel albis demum pallide lilacinis imprimis in saccharo officinaro commixto, floccosis; hyphis aereis ramosis, adscendentibus, septatis, 3μ cr., ramos fertiles brevissimis gerentibus; reverso

incolorato; fructibus conidicis usque 100 longis, e basidiis sessilibus, solitariis vel verticillatis, aut, e ramis brevissimis vel apicibus hyphorum aeriorum, 1, 2, 3 verticillos ramulorum et basidiorum, catenas longas et divergentes conidiorum gerentum; basidiis basidibus incrassatis, apicibus acuminatis et divergentibus, 7-10 longis; conidiis $2.5-3 \times 2\mu$ ellipticis, laevibus, pallide lilacinis.

Coloniis gelatinam lente liquefacientibus, alkalinis lacmo.

Comm., Prof. G. F. Atkinson et C. W. Edgerton, Ithaca, N. Y.

Cultivated in pure gelatin or bean agar white, white to pale lilac in cultures containing sugars, more or less loosely floccose with hyphae branched, septate, ascending, 3μ in diameter, producing conidial masses upon very short branches irregularly distributed, or becoming conidiophores toward the apex; reverse of colony not discolored; conidial fructifications up to 100μ in length, consisting of solitary, sessile conidiiferous cells, or verticils of conidiiferous cells, or short branches bearing 1, 2, or 3 verticils of branchlets and conidiiferous cells with long, tangled chains of conidia. Conidiiferous

cells flask-shaped, divergent at the apices, acuminate, 7-10 μ in length; conidia elliptical, smooth, 2.5-3 by 2μ , thin walled, pale lilac. Colonies slowly liquefy gelatin, with strongly alkaline reaction.

Received from Prof. G. F. Atkinson and C. W. Edgerton, Ithaca, N. Y.

A relationship of this species to the common green forms is very doubtful. The chains of conidia produced break up so quickly and completely in mounting in fluid for examination that it is often difficult to find even a single conidium attached to its sterigma. The hyphae with branches and basidial cells, aside from the production of long conidial chains,

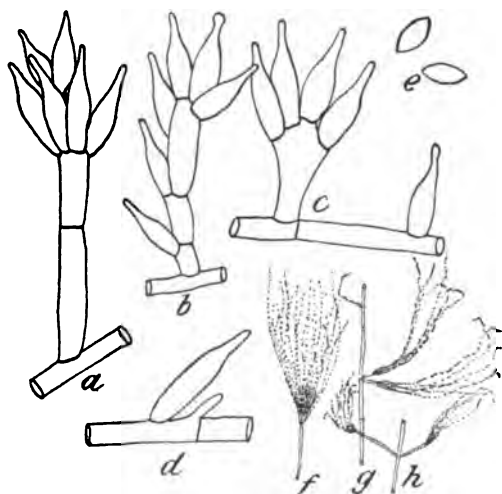


FIG. 30.—*Penicillium lilacinum*: a, b, c, short conidiophores and verticils of conidiiferous cells showing the various branching and arrangement of cells ($\times 1,600$); d, conidiiferous cell, solitary and sessile on an aerial hypha, not uncommon in this species ($\times 1,600$); e, conidia ($\times 1,600$); f, g, h, sketches of conidial fructifications, varying from a single chain to a typical penicillate form ($\times 260$).

might readily be placed in any one of several hyphomycete genera. The form of conidial fructification varies from a single conidiiferous cell or basidium with a chain of conidia upon an aerial hypha to a single verticil, or a branch with two or three successive verticils and even to a terminal fructification allying it with the typical penicillate forms.

CULTURAL DATA.

Color white to a characteristic lilac shade; reverse of colony white; color in media, none.

Odor, none.

Fifteen per cent gelatin in water, fair growth, not heavy, white; liquefaction rather slow—14-16 days; litmus reaction strongly blue. Peptone milk sugar gela-

tin (Conn's), liquefied in 2 weeks, white colonies. Potato plugs, white colony; Cohn's solution, very weak growth but typical lilac color.

Synthetic fluid (Dox's), carbon applied as: Cane sugar 3 per cent to 30 per cent, good growth with typical lilac color, alkaline reaction, no fermentation. Lactose 3 per cent, germination only, slight growth. Lactic acid 0.9 per cent, germination only. Levulose 3 per cent, slow growth. Galactose 3 per cent, slow development with alkaline reaction. Glycerin 3 per cent, not characteristic, little more than germination. Butterfat, typical colony giving brownish color to fluid and causing drops of yellow oil to separate out.

Milk, curdling, slow; digestion, slow; color in milk, none.

At 37° C., best growth; at 20° C., good growth.

PENICILLIUM INTRICATUM n. sp.

Latin diagnosis.—Coloniis in gelatina vel agar phaseoli cultis, albis, griseis, griseo-glaucis, demum griseis, lente fere fuliginis, floccosis; zonatis; parte aerea usque 1-3 mm. cr., ex hyphis aereis ramosis dense intricatis; reverso incolore vel sulphureo interdum lente avellaneo; substrato sulphureo colorato; conidiophoris interdum terminalibus plerumque ex hyphis aereis brevibus 30-50 μ ramosis; fructibus conidiciis 50-100 μ usque 140 μ longis—multo longioribus in substratis saccharinis—ex verticillo basidiorum, vel ex 1-3 verticillis basidiorum in ramis divergentibus, vel ex verticillis ramulorum et basidiorum eodem verticillo, catenis conidiorum saepe columno laxe convergentibus; basidiis 8-10 $\times$ 2-2.5 μ , paucis (4-10) verticillo, cum catenis basidiorum divergentibus; conidiis ellipticis vel globosis, hyalinis, vel pallide glaucis, 2.5-3 μ diam., laevibus, leptodermibus, intus granulosis, in catenis manentibus submersis; coloniis gelatinam nonliquefacientibus, alkalinis lacmo.



FIG. 31.—*Penicillium intricatum*: a, b, c, conidial fructification, conidiiferous cell, conidial chain ($\times 1,600$); d, e, f, sketches of conidiophores, branching and arrangement ($\times 260$).

Cultura ex humo, Prof. W. M. Esten, Storrs, Conn., 1907.

Colonies upon gelatin or bean agar, white, gray, greenish gray, when old gray or smoky, floccose, becoming a mass of interwoven hyphae and ropes of hyphae 1-3 mm. in thickness; reverse of colony and substratum not colored in bean agar, more or less sulphur yellow or even brownish in sugar media; conidiophores sometimes terminal, more commonly branches of aerial hyphae 30-50 μ in length; conidial fructifications 50-100 up to 140 μ in length, or much longer in old sugar cultures, consisting of simple verticils of conidiiferous cells, or of 1-3 verticils upon divergent branchlets, or of branchlets and conidiiferous cells in the same verticil; conidiiferous cells 8-10 by 2-2.5 μ , few (4 to 10) in each verticil, bearing more or less divergent chains of conidia frequently aggregated into a loose column; conidia elliptical or globose, hyaline or pale greenish, 2.5-3 μ diameter, smooth, thin walled, granular within, remaining in chains in fluid mounts; colonies alkaline to litmus, not liquefying gelatin.

* Found in cultures from soil, Storrs, Conn., by Prof. W. M. Esten, 1907.

CULTURAL DATA.

Color white or greenish gray—not green, grayish to brown or drab; reverse and medium uncolored or sulphur-yellow in some media; litmus reaction strongly alkaline. Potato and bean agar, typical. Potato plugs, weak growth, not adapted to this species. Cohn's solution, weak growth, yellowish-green colonies.

Synthetic fluid (Dox's) carbon supplied as: Cane sugar, grew well up to 30 per cent. Lactose 10 per cent, good colonies. Levulose 3 per cent, good growth, yellowish mycelium, fluid yellowish, alkaline. Galactose 3 per cent, typical alkaline reaction. Glycerin 3 per cent, slow growth. Butterfat, good growth, reverse yellow, fat little changed.

Milk, fruiting areas upon glass, mycelium in contact with milk sulphur-yellow; curdling (0.25 per cent calcium chlorid added) very slow—3 weeks; digestion very slow in normal or acid milk, rapid when alkaline; color in milk, none.

At 37° C. grew more rapidly than at 20° C.

***PENICILLIUM SPINULOSUM* n. sp.**

Latin diagnosis.—Coloniis in gelatina vel agar phaseoli cultis, atro-viridibus, demum fere atris, cito et late in substrato crescentibus, margine sterili lata juvenilibus; parte

aeria ex conidiophoris et ex hyphis floccosis sparsis composita; reverso incolorato; conidiophoris 105–300 $\times$ 3–3.5 μ , vel longioribus, apice 5 μ incrassato, verticillum basidiorum 9.5–11 $\times$ 2–3 μ gerente; fructibus conidicis in columno denso 300 usque 500 $\times$ 15–30 μ ex catenis conidiorum compositis; conidiis pyriformibus vel globosis, 3.2–3.5 $\times$ 3.6–4 μ , leptodermibus, primum laevibus demum minutissime spinulosis; coloniis gelatinam lente liquefacientibus, acidis lacmo.

In cultura in laboratorio, Hannover, Germania.

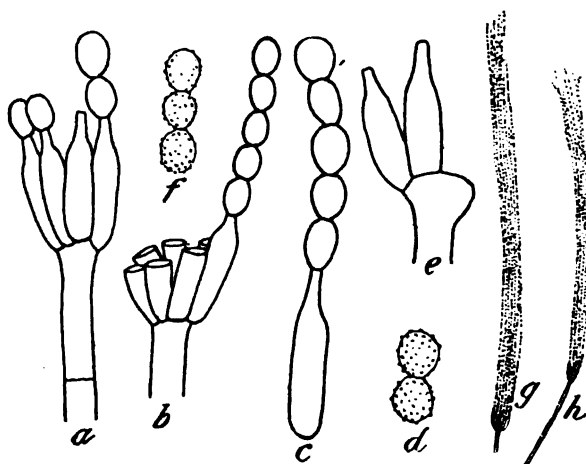


FIG. 32.—*Penicillium spinulosum*: a, b, conidial fructifications consisting of single verticils of conidiiferous cells ($\times$ 900); c, conidiiferous cell with chain of young conidia smooth ($\times$ 900); d, f, ripe conidia, delicately echinulate ($\times$ 900); e, swollen end of conidiophore bearing conidiiferous cells ($\times$ 900); g, h, sketches of conidial fructifications ($\times$ 1,400).

Cultivated upon gelatin or bean agar, deep green, spreading broadly in the substratum with broad sterile margin when young; aerial portion consisting of conidiophores and scattered aerial hyphae; reverse of colony not discolored; conidiophores 150–300 μ or longer by 3–3.5 μ , with apex enlarged to 5 μ in diameter, bearing a single verticil of conidiiferous cells 9.5–11 by 2–3 μ ; conidial fructification a close column of conidial chains up to 300 or even 500 μ in length by 15–30 μ ; conidia pyriform to globose, 3.2–3.5 by 3.6–4 μ , very thin walled, smooth at first then delicately spinulose or verrucose, yellowish green then almost smoky; liquefying gelatin slowly, with strongly acid reaction.

Found as a contamination of another species of *Penicillium* obtained in Doctor Wehmer's laboratory at Hanover, Germany. Easily recognized and cultivated.

CULTURAL DATA.

Color deep dull green; reverse cream, or slight traces of pink or violet; color in media, none.

Odor, none.

Fifteen per cent gelatin in water, slow but typical; liquefaction, rather slow and variable; litmus reaction, acid. Potato and bean agar and potato plugs, typical, producing a very heavy layer of dark-green conidia when cane sugar is added. Raulin's fluid, typical. Cohn's solution, germination only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, grows well up to 50 per cent solution with acid reaction. Lactose 3 per cent, weak growth. Levulose 3 per cent, typical. Glycerin 3 per cent, half normal growth. Potato starch 3 per cent, typical. Butterfat, rich growth.

Milk, colonies grow very slowly; curdling (0.25 per cent calcium chlorid added) very slow; digestion, very slight; color, none.

At 37° C. grew better than check at 20° C.

PENICILLIUM No. 28.

Colonies upon sugar gelatin and potato or bean agar, gray-green with broad white border when growing, floccose, tangled tufts of hyphæ and ropes of hyphæ spreading indeterminately upon the substratum, reverse yellow or tan on media containing sugar, conidiophores arising direct from the substratum as short lateral branches from 38–160 μ in length, 3 μ in diameter, swelling to 5 μ at apex, from aerial hyphæ or ropes of hyphæ. Conidial fructification a simple column, 300 μ or even 500 μ in length by 10–15 μ in diameter, produced from a single whorl of conidiiferous cells at the apex of the conidiophore. Conidia elliptical to globose, 2–3 μ or 2–2.4 μ by 3–3.3 μ in diameter, light yellowish green in mass, smooth. Colonies liquefy sugar gelatin. Litmus reaction strongly acid.

Grows readily upon all common media.

Found at Storrs, Conn., upon decaying mushroom. Very characteristic and readily recognized from others with related morphology.

CULTURAL DATA.

Color gray-green; reverse yellow, or tan when sugar is present; color in media, more or less yellow, according to media.

Fifteen per cent gelatin in water, good growth, clear green; liquefaction slow—2 weeks or more; litmus reaction acid. Potato agar, typical growth, acid reactions. Bean agar, typical growth, acid reactions. Raulin's fluid, good growth, edges pink, fluid brownish fluorescent. Cohn's solution, weak growth.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, good growth up to 50 per cent, acid reaction. Lactose 3 per cent, slow but fairly typical growth. Lactic

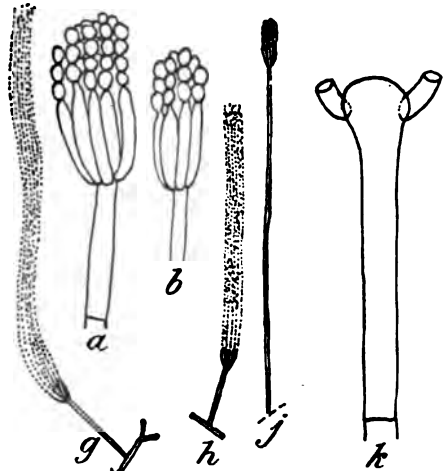


FIG. 33.—*Penicillium* No. 28: a, b, conidial fructifications each a single verticil (X 900); g, h, j, sketches of conidiophores and fructification (X 140); k, tip of conidiophore, swollen at apex, bases of lowest two conidiferous cells (X 1,600).

acid 0.9 per cent, fair growth, not good. Levulose 3 per cent, good but slow colonies, acid reactions. Glycerin 3 per cent, medium growth, pink tinge to fluid. Potato starch 3 per cent, good growth, yellow drops of transpired fluid, fluid tinged yellow at top only. Butterfat, rich growth, fluid reddish brown.

Milk, good growth, acid in litmus milk; curdling (0.25 per cent calcium chlorid added) in 8-9 days, good curd; digestion slow, incomplete; color, pale yellow in digested fluid.

At 37° C., killed; check at 20° C., good.

SPECIES FORMING PINK SCLEROTIA.

Four races have been found in which pink sclerotia are regularly formed in culture. These sclerotia are elliptical to globose and from 200 to 500 μ in diameter. They begin to be formed within the first week in richly nourished cultures. Although examined repeatedly, no trace of ascus formation has yet been found. These forms are included here under their serial numbers, 29, 30, 31, and 32, rather than with specific names. The descriptions and figures introduced will, it is thought, identify these organisms clearly in their *penicillium* form, but the uniformity of sclerotium production makes ascus production so probable under proper conditions that it seems best not to give specific names to this imperfect form when some of them may be already recognizable by others or by further investigation.

PENICILLIUM No. 29.

Colonies grown upon gelatin and potato or bean agar white to gray-green, sometimes partly clear green, becoming zonate with rings of pink sclerotia in age, sparsely or loosely floccose, indeterminate broadly spreading margins persistently white, slightly yellow below. Conidiophores 80-200 μ or even 400 μ by 3-5 μ commonly 150-200 μ in length as branches, usually perpendicular, from hyphæ 4-5 μ in diameter. Conidial fructification a single verticil of rather few (about 12-15) conidiferous cells 9 by 2 μ , producing chains of conidia in a loose column 150-250 μ or even 400 μ by 20-30 μ . Conidia elliptical, 3-3.6 by 2.3-2.8 μ , smooth, very pale blue (transmitted light). Sclerotia in loose networks of mycelium, numerous, pink, elliptical to globose, 150-300 μ in diameter. These begin to appear in one week in gelatin cultures. Colonies liquefy sugar gelatin slowly but completely in 10-12 days. Give a strong acid reaction with litmus media.

Characterized by the production of large numbers of pink sclerotia with comparatively small quantities of conidia, whereas the next form (*Penicillium* No. 30) produces few sclerotia and great quantities of conidia.

Collected at Storrs, Conn., on decaying mushroom. Probably not closely related to the common species of *Penicillium*, but its occurrence in culture and ready adaptation to all media tried, in numerous cultures, justify its inclusion with these species.

CULTURAL DATA.

Color white to gray or green, with many pink sclerotia; reverse colorless or slightly salmon; color in media slightly yellow in some media.

Odor, none.

Fifteen per cent gelatin in water, good growth; liquefaction, 15 days; litmus reaction alkaline. Potato agar and bean agar, typical colonies, white or gray, with few green areas and abundant pink sclerotia. Raulin's fluid, typical. Colonies upon

media with cane sugar produce sclerotia more numerous and more quickly (5 days) than without sugar. Butterfat as a source of carbon in Dox's fluid, typical colonies.

Milk, typical; curdling (0.25 per cent calcium chlorid added) in 9 days; digestion, slow; color in milk, none.

•
PENICILLIUM No. 30.

Colonies on sugar gelatin and potato or bean agar gray-green or green persistently, surface growth mostly of crowded conidiophores and producing pink sclerotia at the surface or partly embedded in the substratum 150–300 μ in diameter, broadly spreading. Conidiophores from 240–525 μ , usually about 300 μ , in length either arising separately from the substratum or as branches very close to its surface. Conidial fructification a single verticil of conidiiferous cells bearing conidia in a close column up to 500 μ in length by 15–30 μ . Conidia elliptical or subglobose 2.5 by 3 μ or 3 μ , with a slight greenish color. Colonies liquefy sugar gelatin rather slowly, and give an acid reaction with litmus.

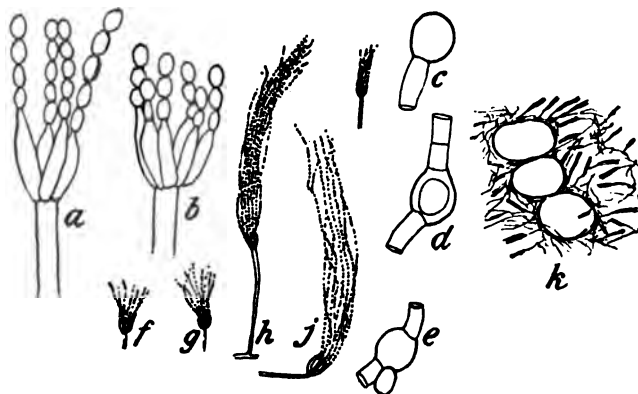


FIG. 34.—*Penicillium* No. 29: a, b, conidiophore and verticil of conidiiferous cells ($\times 900$); c, d, e, germination of conidia ($\times 900$); f, g, h, j, sketches of conidial fructifications ($\times 140$); k, diagrammatic sketch from photomicrograph showing relations of sclerotia and conidial fructifications.

Apparently related to No. 29, but differing in the length and density of its column of conidia, in the position of the sclerotia, in habit, in culture, and in its acid reaction.

Collected at Storrs, Conn., upon decaying *Laccarius vellereus*, September, 1904.

CULTURAL DATA.

Color green or grayish green, persistently, with abundant pink sclerotia; reverse uncolored; color in media, none.

Odor, none.

Fifteen per cent gelatin in water, typical; liquefaction rather slow; litmus reaction acid. Potato agar and bean agar, typical. Potato plugs, typical. Cohn's solution, germination only.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, rich growth. Lactose 3 per cent, slowly typical colonies, acid reaction. Lactic acid 0.9 per cent, weak growth. Levulose 3 per cent, medium growth. Galactose 3 per cent, typical. Glycerin 3 per cent, very weak growth. Butterfat, slow, but characteristic colonies.

Milk, curdling (0.25 per cent calcium chlorid added) in 9 days; digestion, slow but complete; color in milk, none.

At 37° C., no growth; grew when cooled; check at 20° C., typical.

PENICILLIUM No. 31.

Colonies upon gelatin and potato or bean agar from white to gray to gray-green mostly white, with few areas of green conidia sprinkled with pink sclerotia, sparsely floccose, broadly spreading. Conidiophores branching from aerial hyphæ, very short to 380μ in length, commonly $150-240\mu$, conidial fructification with a single verticil or once branched with branch, conidiiferous cells and chains of conidia divergent, up to 140μ in length, but usually much less. Conidia $2.5-3\mu$ globose, smooth, rarely found in quantity to color the colony. Sclerotia elliptical or globose, $160-330\mu$, pink, developed in 10-15 days. No asci have been secured. Colonies liquefy sugar gelatin rapidly and give a strongly alkaline reaction to litmus in the same cultures.

Grows readily in conidial transfers upon all common media.

Collected upon decaying *Clavaria* at Storrs, Conn., September, 1904. Identical culture sent from Cambridge, Mass., by Dr. A. F. Blakeslee in culture obtained from fruit imported from Porto Rico.

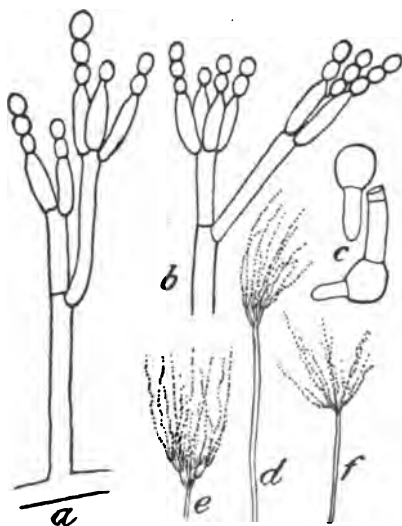


FIG. 35.—*Penicillium* No. 31: a, b, branching of conidiophore ($\times 900$); c, germination of conidia ($\times 900$); d, e, f, sketches of conidiophores.

CULTURAL DATA.

Color white or gray, conidial areas gray-green, very numerous pink sclerotia; reverse colorless or with yellow areas; color in media, none or slightly yellowish. Odor, none.

Fifteen per cent gelatin in water, typical white or gray colonies; liquefaction rapid; litmus reaction alkaline. Potato and bean agar, typical, cultures with sugar added become distinctly greener than others. Potato plugs, typical, white or gray with greenish areas, sclerotia, and crystal drops of transpired fluid. Raulin's fluid, some growth, not entirely typical. Cohn's solution, weak development but characteristic.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar 1.5-20 per cent, typical growth. Lactose 3 per cent, weak growth. Lactic acid 0.9 per cent, no growth. Levulose 3 per cent, typical. Galactose 3 per cent, typical, alkaline. Glycerin 3 per cent, slight growth. Butterfat, typical growth.

Milk, curdling (0.25 per cent calcium chlorid added) in 9 days; digestion complete; color, none.

At 37°C ., no growth, grew when cooled; check at 20°C ., typical.

PENICILLIUM No. 32.

Colonies upon milk-sugar-gelatin and potato or bean agar gray-green; floccose, but with aerial part mostly long conidiophores and few vegetative hyphæ, slightly yellowish to pronounced salmon color below; broadly spreading; developing elliptical to globose sclerotia $150-200\mu$ in diameter at the surface of the substratum in 2-3 weeks. Conidiophores $200-500\mu$ by $3-4\mu$. Conidial fructification a verticil of 3-5 branches $10-17\mu$ by $2-3\mu$ rarely a secondary verticil, each bearing a dense verticil of conidiiferous cells, $8-10\mu$ by 2μ producing long, parallel, or slightly divergent chains of conidia. Conidia elliptical or fusiform, $3.5-4\mu$ by $2-3\mu$, green, granular within, smooth, swelling in germination to 6μ and producing from one to several germ tubes. Colonies slowly liquefy milk-sugar-gelatin and produce purple or neutral colors in litmus media.

Sent by Prof. P. H. Rolfs from Miami, Fla., upon portion of pineapple, March, 1905.

CULTURAL DATA.

Color gray-green, with scattered white to pink sclerotia; reverse, sulphur-yellowish to pronounced salmon; color in media reddish or yellowish in special cases, others none. Odor, none.

Fifteen per cent gelatin in water, typical; liquefaction, none in 15 days, later very slow liquefaction; litmus reaction neutral, leaves both acid and alkaline media purple-blue. Potato agar and bean agar, typical, slightly thinner than gelatin cultures, gray-green without sugar, clear green with cane sugar. Potato plugs, typical, transpires yellow drops which become very dark yellow (balsam). Raulin's fluid, good colonies becoming rosy below. Cohn's solution, small colonies, fluid slightly yellow.

Synthetic fluid (Dox's), carbon supplied as: Cane sugar, grew in solutions up to 30 per cent with acid reaction. Lactose 3 per cent, very slow growth of small characteristic colonies. Lactic acid 0.9 per cent, good growth, light green. Levulose 3 per

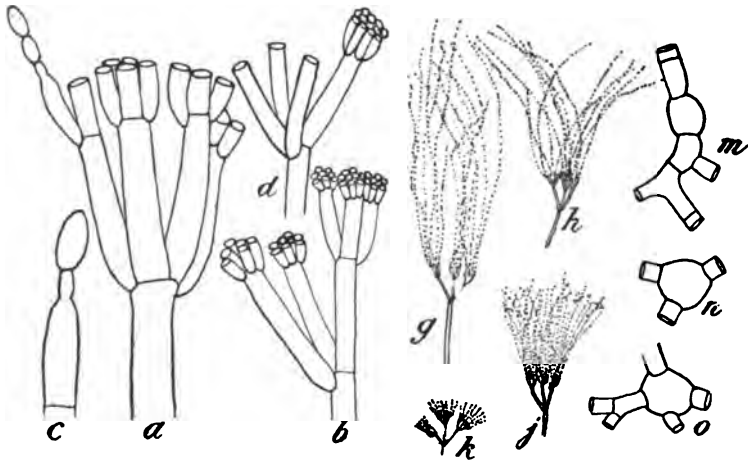


FIG. 36.—*Penicillium* No. 32: a, b, d, branching of conidial fructifications (a $\times 1,400$, b and d, $\times 900$); c, a single secondary verticil ($\times 1,400$); g, h, j, k, sketches of fructifications of various ages ($\times 140$); m, n, o, germination of conidia ($\times 900$).

cent, very slow-growing but heavy colonies. Galactose 3 per cent, typical. Glycerin, very small colonies. Butterfat, typical colonies.

Milk, curdling (0.25 per cent calcium chlorid added) very slow; digestion, very slow; color in milk, none.

At 37° C., grew more rapidly than check at 20° C.

COMPARATIVE CULTURAL DATA.

A summary of accessory cultural data has already been given for each species in connection with the descriptions. Many series of cultures have been made with numerous media to obtain data as to the ability of the species studied to grow upon particular media or under particular conditions. It has been possible thus to determine the relative activity of single species and groups of species. Although particular species in these cultures have shown unique differences which assist in their differentiation, the most valuable

result of comparative culture is found in the separation of the series into groups of races or species which resemble each other closely in their metabolic activities. Part of these experiments are tabulated in Tables 2, 3, 4, 5, and 6, and will be discussed in the following sections.

Since the complex composition of the media in common use for cultural work makes the analysis of the data obtained impossible, it was first necessary to determine the reactions of these species to some of the individual substances of which these media are composed. It has already been noted that gelatin alone in distilled water sustains growth in the large majority of the species of this genus. These cultures in certain species lack green color, which, however, becomes present on using the peptones and sugars added to gelatin in most formulæ. Such media are still too complex to make close analysis of results possible.^a

Many of the determinations given were made in duplicate and in some cases the entire series was repeated one to several times. In inoculating cultures for this work conidia were transferred to the tubes in large numbers, so that their presence could be detected by examination with a lens. Where species failed to grow, the doubts of inoculation were commonly dispelled by the addition of cane sugar, which permitted the conidia to develop normally if present and still viable. The presence of germinated conidia upon the surface of a medium is good evidence of proper inoculation. The data given are believed, therefore, to represent with a fair degree of accuracy the comparative cultural reactions of the species used.

In reporting these series of comparative cultures, the data have been tabulated as far as possible for convenient comparison of the relative activity of the different forms. (See tables beginning on p. 98.) The names as far as determined are given, together with the cultural number, in Table 1. In the remaining tables the numbers are repeated without the names. In studying the tables a reference to cultural numbers will quickly locate the forms discussed.

CULTURES IN DISTILLED WATER.

To determine the possibilities of growth from food stored in the conidia, cultures were made in distilled water. Of forty-four strains under cultivation but six showed clearly discernible germination. None produced more than germ tubes hanging down into the fluid.

^a This work was carried on in cooperation with Mr. A. W. Dox, who has studied the metabolism of the species concerned with cheese ripening (*P. camemberti* and *P. roqueforti*), as well as a few other species, under many conditions of culture, while the writer has conducted comparative studies of a large number of forms under more limited cultural conditions. All chemical questions arising throughout this work have been passed upon by Mr. Dox.

AGAR-AGAR AS A SOURCE OF FOOD.

Tubes of 1.5 per cent agar-agar in distilled water were inoculated with eighteen species of *Penicillium*. Sixteen of these produced growth. In all cases the colonies produced were very small, some of them barely discernible to the naked eye. Not one of them was distinctly colored by conidial masses, but in nearly every case some conidial fructifications were found. These cultures show that the species tested were able to obtain from the medium sufficient nourishment for very slight growth.

AGAR-AGAR AS A SOURCE OF CARBON.

Agar-agar is a carbohydrate and might serve as a source of carbon if other nutrients were supplied. One and one-half per cent of agar was therefore introduced into Mr. Dox's synthetic fluid, already containing all essential elements except carbon. Thirty-seven races of *Penicillium* were inoculated into this medium, and nearly every species produced some growth. Examined with the microscope, conidial fructifications were found in nearly every case, but in no case was the colony large enough or definite enough to affect the observer's estimate of results if such growth were added to or subtracted from the colonies upon nutrients really adapted to sustain the species studied. Dox's stock solution, with or without the addition of agar, was in this way shown to be a safe medium for the study of the metabolic reactions of these species to changed sources of carbon.

The possibility of error in the introduction of agar was shown in the following manner: One and one-half per cent of agar was introduced into Dox's fluid and 1.1 normal lactic acid added in quantity to make the whole 0.5 per cent acid. The medium was then autoclaved. After this treatment the agar refused to solidify. Tubes of this fluid were inoculated with nine different species of *Penicillium*. All except *P. brevicarule* grew well and produced colonies recognizable by their cultural characters. In introducing agar in such work it is therefore necessary to guard against the introduction of acid before dissolving the agar, since this changes the agar itself into other carbohydrates assimilable by fungi. Although parallel cultures were commonly made with agar, the studies of metabolism recorded in this paper were made in tube culture of the fluid nutrients only, to avoid possibilities of error.

VARIOUS SOURCES OF CARBON.

Cane sugar (Tables 4 and 5).—Cane sugar was added to Dox's fluid in the following percentages: 1.5, 3, 10, 20, 60, and 75. Of the species used, but one—*P. digitatum* of Saccardo—failed to grow typically. This, together with other work, indicates that this species is

incapable of assimilating nitrogen from the sodium nitrate of this solution. Four more forms—*P. brevicaulis* and its closely related varieties, and *P. roseum* Link—reached normal appearance slowly. Other cultures indicate that these forms assimilate nitrogen in this form less readily than in organic combinations. This medium containing cane sugar in amounts as great as 20 per cent proved well adapted for all other species tried. The medium as used was neutral or slightly alkaline in reaction. Thirty-three of the forms inoculated into it produced pronounced acid reactions to litmus in a few days, i. e., were able to ferment this form of sugar. In prolonged culture with smaller amounts of sugar, some of these forms finally reduced the acidity and even brought about an alkaline reaction again; others remained acid as long as observed. Twelve forms failed to produce acidity. This number includes the four forms in which growth was delayed and *P. italicum*, *P. luteum*, *P. purpurogenum*, *P. roqueforti*, and *P. duclauxi*.

In cultures at a concentration of 60 per cent cane sugar, five forms produced typical colonies at once; six others slowly reached normal proportions; a few more grew fairly well; but fully half the species tried produced germination with but little further growth. Water in amount approximately to reduce the concentration to 35 per cent was added to the cultures that failed to produce normal colonies, and this was followed by the prompt recovery of several species which quickly reached normal development. Critical examination of the data obtained showed that closely related types responded in exactly the same manner to changed percentages of cane sugar. As a means of separating closely related forms, further determination seemed fruitless. Exact determination of the maximum percentages of cane sugar tolerated by particular species has not been completed. The inhibition is not a stoppage of all development at a definite critical concentration, but rather a gradual reduction of activities with the increasing concentration of the medium. Specific maxima and minima are therefore almost impossible to define. All determinations would therefore rest upon the judgment of the observer rather than upon fixed standards.

Lactose (Tables 4 and 5).—Lactose was added to Dox's fluid in percentages up to 10 per cent. Prompt and normal development was determined in eight forms; nine more forms reached typical appearance more slowly. Only twelve of these produced definite acid reactions to litmus. Among the forms included in the seventeen, four groups of closely related organisms were found, namely, the *camemberti* group, Nos. 5, 6, 39; the common green group, Nos. 22, 23, 40; the *chrysogenum* group, Nos. 25, 26, 35, and 44; and the *brevicaulis* group, Nos. 2, 3, and 4. With lactose as with cane sugar closely related forms give

approximately the same reaction in most cases. Three of these groups show acid and the fourth alkaline tests with litmus. A few of the remaining species continued to grow until after several weeks they reached almost normal development. In such cases the assimilation of carbon from lactose seems to be very slowly and with difficulty accomplished by a large proportion of the species studied, and to be practically impossible to some species.

Comparison of the litmus reactions produced by the various species with cane sugar and with lactose accounts for striking differences in this reaction when litmus is introduced into gelatin or agar media containing these two forms of sugar. A species which will ferment cane sugar and not ferment lactose will produce an acid reaction with one and an alkaline reaction in media containing the other.

Lactic acid (Tables 4 and 5).—Tubes were prepared containing Dox's fluid to which 0.9 per cent of lactic acid was added. Thirty forms were inoculated into this medium. Of these forms nine produced normal and typical colonies, showing but slight inhibiting effect from the acid. As many more cultures slowly became typical colonies. Nearly every form germinated and produced slight growth. In the *camemberti* group (Nos. 5, 6, 39) and some others the litmus reaction became alkaline. It was thus shown that a series of species could secure carbon from lactic acid and in doing so destroyed the acid character of the medium. The species which grew most rapidly in lactic acid were those which had developed best in the lactose solutions.

Levulose (Tables 4 and 5).—Tubes were prepared into which 2.5 per cent of levulose was introduced as the source of carbon. The results as tabulated may be seen to group together about the same species as the previous experiments, except that one or two forms were found to grow well in levulose that failed to grow with lactose or lactic acid as a source of carbon. Fourteen forms produced typical colonies without inhibiting effects, while five more slowly reached typical development.

Galactose (Tables 4 and 5).—A series of cultures was made in the same way with 3 per cent galactose as a source of carbon. Galactose proved much better adapted to supply carbon than either lactose or levulose. Twenty-five forms produced typical growth, and others grew more or less readily. This form of sugar therefore proved of but small assistance in the separation of species.

Glycerin (Tables 4 and 5).—Cultures offering carbon in the form of 3 per cent glycerin produced much less growth than those containing sugars. Eleven forms eventually reached fairly typical growth; four only of these showed no restraining effect of the medium. Of these, three are probably closely related if not merely races of a single species—the one most common in general cultural work in this

region. Apparently glycerin presents a form of carbon much less available for assimilation by species of this genus than the sugars.

Butterfat (Table 4).—To test the ability of these fungi to assimilate fat, butter was melted, strained, filtered through filter paper, and added to Dox's fluid. Although not chemically pure, perhaps, it is believed that the amounts of other nutrients would be too small to affect results. Only one form (*P. digitatum*) failed to grow. *P. luteum* gave only slight growth. The majority of forms, although growing slowly, produced typical or fairly characteristic colonies. The masses of fat were visibly much changed, becoming incrustated with a white substance in most cultures. In a few cultures the action of fungus caused the separation of the various fats, so that drops of yellow oil separated out from the remaining nonliquid matter.

Potato starch.—In one series twenty-seven forms were cultivated in Dox's fluid, containing 3 per cent of potato starch. All of the common species of the genus were found to grow normally upon a medium containing starch as the source of carbon. The characters in this medium were approximately the same as in the stock agar or gelatin cultures. Two species (*P. decumbens* and *P. digitatum*), which failed to grow well have since been shown to depend upon the presence of cane sugar for vigorous growth and green color to their spores. Similarly the same species grown upon plugs of potato failed to produce strong colonies of pronounced green color.

Malic and succinic acids.—Series of cultures were made with 1 per cent malic acid and with 1 per cent succinic acid as sources of carbon. All species germinated, but no species reached fully typical development in either series. Some few species produced slowly colonies of half or more of the normal size with conidial masses of typical color. Many of the species grew sufficiently to produce a few conidial fructifications recognizable with the hand lens. These two series emphasize the observation already made that species inoculated into a medium ill adapted to their nourishment will nevertheless grow and produce small amounts of fruit under widely different conditions even where normal growth is impossible.

CULTURES IN RAULIN'S FLUID AND COHN'S SOLUTION.

The comparative data for cultures in Raulin's fluid and in Cohn's solution are shown in Tables 3 and 5. Raulin's fluid, as given by Smith, is a highly acid medium and has been found very well adapted for the growth of certain species. The solution is, however, too complex to make analysis of cultural results upon it readily possible. It contains carbon in three different forms—tartaric acid, potassium carbonate, and magnesium carbonate—and nitrogen in two forms

in ammonium nitrate. Aside from data as to growth or failure to grow, culture in such a medium is fruitless.

Cohn's solution is also an acid medium, but its acidity is due to the presence of monopotassium phosphate (KH_2PO_4). In a series of cultures with Dox's formula it was shown that the monopotassium phosphate had practically no different effect upon cultures of these species than the dipotassium phosphate (K_2HPO_4).

Very few of the species were found to grow typically upon Cohn's solution, however, although nearly all germinated. Comparative work with Dox's formula shows that but few of the species studied are capable of normal assimilation of carbon from forms of tartaric acid. Only three of the species experimented with failed to show distinct germination. One of these inoculated with *P. camemberti* var. *rogeri*, remained four months without exhibiting any germination of the conidia. The conidia were then transferred with a platinum needle to a petri dish of gelatin; under these conditions the same spores developed into typical colonies without showing any ill effects of four months' immersion in the fluid which they were unable to assimilate.

In solutions nontoxic in character many species exhibit definite selective preferences for nutrient elements in particular chemical combinations. This selective adaptability to particular forms of nutrients differs greatly for different groups of species. Some forms of wide distribution seem adapted to produce typical growth upon quite varied chemical solutions. Other species (e. g., the rots of citrus fruits), equally wide in their distribution, are closely dependent upon particular forms of food, whereas between these extremes are many forms exhibiting preferences as to nutrients yet capable of development, although more slowly, in media containing nutrients in forms assimilated with difficulty. These experiments have offered no tangible evidence of rapid adaptation to media found unadapted at first to development. Such power of gradual adaptation is not excluded, however, by these experiments.

COLOR IN CONIDIAL AREAS.

In dealing with all species it has been found by repeated experiment that the green color of the spores is dependent upon the proper assimilation of the carbon element. Many of these species produce a brighter green when cane sugar is present than with any other form tried. Grown upon gelatin, or upon potato agar or bean agar (free from sugar), several species produce green masses of conidia which rapidly become some shade of gray, brown, or almost black in media without sugar, but when cane sugar is present they are deep green and remain unchanged for much longer periods. Certain other species entirely lack green color except when sugar is present.

A color determination is therefore dependent for its value upon knowledge of the form of carbon presented by the medium. These same species grown with lactose as a source of carbon respond exactly as they do in potato or bean agar or in gelatin free from sugar. Another series of forms are little if at all changed in color by changing the form of carbon presented, provided only that they are able to grow readily in the medium as presented. It seems therefore certain that very many widely distributed species are capable of assimilating carbon in very widely different chemical forms and to produce in such cases normal and typical growth and colors, whereas other species are entirely dependent for normal growth and color upon the presence of particular chemical combinations or groups of combinations. Study of the forms so responding likewise shows that the ubiquitous species are capable of assimilating carbon in the most varied combinations, whereas the forms lacking this power are mostly less common and more specialized.

EFFECT OF CONCENTRATED MEDIA.

Similarly great differences in the mass of the growth produced are directly attributable to the presence of nutrients easily available or in greater concentration. The formulæ usually recommended contain the nutrient used, in extremely dilute proportions. The determinations already given with different percentages of cane sugar show that these species are able to assimilate sugar in widely different concentrations. With the majority of forms studied there seems to be no deleterious effect from increasing the concentration of the nutrients offered until the solution has attained an osmotic pressure sufficient to inhibit growth by plasmolyzing the cells, or until the reduced percentage of water gradually reduced the rate of fungous growth. In one experiment nine species were inoculated into a medium containing 5 per cent cane sugar, 10 per cent Witte's peptone, and 5 per cent Liebig's extract. All species grew luxuriously. The mass of mycelium produced was much in excess of the results with ordinary culture media. One form normally producing sclerotia produced a very rich growth of mycelium, but no sclerotia. In media of higher concentrations the amount of growth—the mass of mycelium and conidia—is, however, so greatly increased as to render the discrimination of the character of the species more difficult by mechanical interference due to the quantity of material. For purposes of study, therefore, the usual formulæ really produce the more satisfactory growth in nearly every species, although from the standpoint of fungus development such media must be recognized as far below the optimum concentration for the species of this genus.

It must further be noted that in cultures containing cane sugar many species continue to produce conidia for a much longer period

than in solutions lacking sugar or some nutrient equally assimilable. In these cultures the quantity of conidia produced increases enormously, often becoming a layer over the whole surface of the colony half a millimeter or more in thickness. The presence of such masses of spores greatly complicates the study of the structure of the colonies, but is especially characteristic of such species.

THE GROUPING OF SPECIES.

Analysis of the cultural tables presented in the light of many series of comparative cultures makes possible the grouping together of particular races or species which possess common cultural characters. It is comparatively a simple matter to single out first the unique forms—those which are never green, those which produce a particular form of sclerotia, those which regularly produce prominent coremia, or those which produce striking colors in the substrata, even those associated with particular substrata. There remain, however, the large number of green forms which lack these striking characters. This large group comprises probably most of the forms which have masqueraded under the name *P. glaucum*. Lines of differentiation among these forms are more or less obscure. Kept in continuous culture races are easily differentiated with the eye by shades of color or habit, but characters of easily recognizable diagnostic value in written descriptions are more difficult to find. Inspection of cultural data shows, however, that there is a well-marked group of these forms which are able to ferment lactose as well as cane sugar. These comprise the *camemberti* group (Nos. 5, 6, 39), the *chrysogenum* group (Nos. 25, 26, 35, 44), and what we may call the “commune” group (Nos. 22, 23, 40). Among those not causing an acid reaction in lactose cultures is a series of rapid liquefiers of gelatin which have many characters in common (Nos. 12, 15, 24, 37, 38). All of these forms show special adaptability to growth in cane-sugar media. No. 15, *P. citrinum*, produces brilliant lemon color, especially in sugar media; No. 38 is given as *P. atramentosum*, from its blackening of the substrata in sugar media and in milk; No. 37 by developing green color when sugar is present, which color is lacking or evanescent without sugar. These forms differ in their color reactions in the medium, in the size and shape of their conidia, in the length and origins of their conidiophores, and in the arrangement of the elements in the conidial fructifications.

Another marked habit difference which holds true throughout many series of cultures is the tendency of colonies of certain species to spread rapidly over the whole surface of the substratum, whereas others are quite restricted in their habit of growth. In the first the developing margin is almost uniformly broad and while growing white, e. g., *P. roqueforti*, *P. italicum*, *P. chrysogenum*, *P. divaricatum*,

P. spinulosum, *P. expansum*, *P. brevicaulis*, and such floccose forms as *P. camemberti* and *P. biforme*; the species of restricted habit with narrow growing border are represented by *P. citrinum* and *P. atramentosum* and their allies. The designation "spreading," "restricted," "with broad margin," or "with narrow margin" is descriptive for colonies of such species.

ODORS.

Although many persons seem to detect the presence of mold by its odor, very many of the species give but little definite odor. A few forms, some of them (e. g., *P. biforme*) always, others upon special media, produce definite odors by which they can be recognized or placed in particular groups. Grown upon gelatin media, *P. brevicaulis* produces a strong ammoniacal odor. A piece of moistened litmus paper held over such a colony will promptly give the alkaline reaction. This organism is recorded as emitting arsine from cultures containing arsenic in any form and it is said to be a very delicate test for the presence of that element^a (Gosio). The two forms here described as varieties of *P. brevicaulis* give exactly the same odor. In nearly every medium used *P. expansum* (the apple rot) gives a strong odor, which suggests decaying fruit to some. Once well distinguished, the presence of this organism can be detected even as a contamination wherever it occurs, by the odor alone. Another given here as *P. atramentosum* produces a very characteristic odor while digesting milk, defined by one as the odor of rancid walnuts, to another it suggested mice. A series of forms when grown upon cane sugar produce a very characteristic odor—an ester, according to the chemists to whom it was submitted—recognizable to the sense of smell, but not definable. The olive-colored orange rot (*P. digitatum*) gives this most strongly, but it is also given by *P. italicum*, *P. decumbens*, and No. 13. The odor of *P. claviforme* is found under nearly all cultural conditions, and would readily identify it were its big coremia not already very distinctive. The common and undefinable green group contains a series of races or forms, many of which give what is popularly called the smell of mold. In others of this group this odor is scarcely distinguishable.

ANAEROBIC CULTURES (WITH CARBON DIOXID).

The possibility of some species developing under anaerobic conditions was tested as follows: Vials were prepared with Dox's fluid having 5 per cent cane sugar as a source of carbon. This had already been shown to be an excellent medium for the growth of nearly all the species used. These vials, containing all the species herein described, were packed in a crate or test-tube basket and put into a Novy jar. The jar was then given in charge of Mr. Dox. The air was exhausted

^a Gosio. Azione di alcune muffe sui composti fissi d'arsenico. Rivista d'Igiene e Sanita Pubblica, Rome, 1892. See page 201.

as completely as possible. Carbon dioxid washed through water and through sulphuric acid was carefully introduced. The exhaustion was repeated and the gas introduced a second and a third time. The cultures were then permitted to stand one week and examined. A culture of *Oidium lactis* among the species of *Penicillium* was found to have grown some. No species of *Penicillium* had produced a colony. The carbon dioxid was then three times exhausted and replaced by air and the jar permitted to stand a second week. Normal colonies of every species were produced. Clearly, therefore, no one of the species of *Penicillium* under experiment was capable of growing in an atmosphere of carbon dioxid, and no species was killed by exposure to such atmosphere.

INCUBATION TESTS.

The importance of temperature in determining the distribution of fungi in nature, and in controlling their presence in the household, the dairy, and the storage room, made the determination of the limits of temperature for the growth of these species desirable. The following incubation experiments were therefore made. (See Table 6.)

1. Incubation at 20° C. This was repeated several times with fully checked records.

2. Incubation at 37° C. (range of variation 35° to 38° C.) for six days; cultures then examined, recorded, and the incubator cooled to 20° C. for the succeeding six days.

3. Use of the ice thermostat.^a Four series of cultures were made in bean-agar with 5 per cent cane sugar—a medium adapted to produce typical colonies of all species in a minimum of time. The temperature of the incubator was recorded eight times a day at three-hour intervals. The range of temperatures in the compartments used and the average of fifty-five observations taken in the first seven days were as follows:

Compartment 1, average 1.05° C., range 0.5° to 2° C.

Compartment 2, average 4° C., range 3.2° to 6° C.

Compartment 3, average 7° C., range 6° to 10° C.

Compartment 5, average 8.7° C., range 7° to 10.5° C.

Observations upon the cultures were made by the writer at 3 and 7 days and repeated, by the kindness of Miss Lucia McCullough, of the Bureau of Plant Industry, at 15, 23, and 29 days. For convenience of comparison, the notes from all these observations have been reduced as fairly as possible to a decimal code, in which germination of conidia without further growth is given as 0.1 and typical colonies at 1.0; fractions from 0.1 to 0.7 represent vegetative mycelium without colored conidial areas, and from 0.7 to 1.0 the

^aBy the courtesy of Dr. Erwin F. Smith the ice thermostat of the Bureau of Plant Industry of the United States Department of Agriculture was placed at my disposal. It was iced and regulated under his instructions.

completion of the typical colony. The figures are brought together in Table 6.

Data at 20° C.—The data at 20° C., as given in the first column of Table 6, are regarded as typical for the species studied and given as 1.0. Numerous series of cultures with all these forms under close observation in the incubator at 20° C. and in the laboratory where the temperatures used ranged from 15° to 25° C. or slightly higher have given approximately the same results. Within these limits, rise or fall in temperature affects the amount of growth or the stage of development of the colonies within a specified time without affecting the character of such growth. The differences between cultures grown at different temperatures within these general limits are quantitative, not qualitative. Unless made for a specific purpose cultures of these fungi may be safely grown outside the incubator without affecting their character, since the conditions in the ordinary working room are approximately those furnished to these forms by nature.

Data at 37° C.—At 37° C. thirteen forms showed normal development. Of these seven grew better at 37° C. than at 20° C., this number including but one well-known species—*P. luteum*. At the same temperature the spores of seven species were killed, including among these *P. italicum* and *P. digitatum*, the species destructive of citrus fruits. Of the green forms abundantly found, only one grew well at 37° C.—*P. chrysogenum*. The numerous green forms studied were not killed, but simply prevented from growing by the heat. Every form except those noted as killed developed normally in the same tubes as soon as cooled to 20° C.

Ice thermostat.—In compartment 1 of the ice thermostat, ranging from 0.5° to 2° C., 20 of the forms experimented with either produced germ tubes only or failed even to germinate in twenty-nine days. Of the remaining 18, only 6 produced colored conidial areas in that time. Several other species produced considerable masses of white mycelium.

In compartment 2, ranging from 3.2° to 6° C., with an average slightly above 4° C., 16 or 17 still showed germination only or complete inhibition; 11 showed colored conidia; several additional forms had germinated or produced distinguishable mycelium.

In compartment 3, with an average temperature about 7° C. and a range from 6° to 10° C., 16 forms produced colored fruit. Of these, 9 had reached the typical appearance of mature colonies of the species within the 29 days. Nine only remained without showing some mycelial growth in addition to germination.

In compartment 5, averaging about 9° C. and ranging from 7° to 10.5° C., all species had germinated and all but one had produced mycelium; 25 forms had developed colored conidia; 17 had produced colonies of typical appearance.

Study of the figures given shows a progressive increase in growth from the coldest to the warmest compartment. Examination of the cultures showed that at the lower limits of growth very many species produce colored fruit very slowly at temperatures at which vegetative mycelium is still developed quite rapidly. There often results, therefore, in cold temperatures a disproportionate growth of white mycelium and a tardy development of colored conidia, which often affects the appearance of the resulting colony considerably. Cultures grown under such conditions would be difficult to identify in many cases. The forms which grew most rapidly at 37° C. either failed to grow at the colder temperatures or responded very slowly. The large majority of the species, and especially those most commonly found in food materials, are seen to begin fairly rapid growth at temperatures within a few degrees of the freezing point. When taken from the ice thermostat all cultures which had failed to produce normal colonies grew quickly to typical appearance. There was, therefore, no injury attributable to continuation for 29 days at low temperature. The general effect of low temperatures upon these species is the suspension of or the reduction of the rate of development. In many cultures the beginnings of growth were difficult to detect and in most cases an exactly critical temperature is not determinable, since cultures not showing any growth in one week seem gradually to adjust themselves to conditions and produce mycelium in the succeeding weeks.

Eustace⁶ has recorded that one of these species (*P. expansum* Link of this paper) will produce rot in storage apples where the temperature of the room as recorded does not rise above 32° F. (0° C.). When the time was extended to two months Petri-dish cultures under the same conditions produced small colonies. The experiments here recorded tend to suggest that very little growth will occur in most species at temperatures nearer than 2° C. to the freezing point, although many of them will germinate. Unpublished records of the temperatures of apples in storage, furnished by Mr. C. D. Jarvis, of this station, showed that the flesh of apples in storage was constantly from 1 to 2 degrees at least above that of the room. Allowing for the conductivity of the thermometer itself, the difference is probably somewhat greater. Both series of data indicate, however, that storage temperature to exclude fungous growth must be close to the freezing point. It is clear that low temperatures (above freezing) merely restrain growth, not entirely prevent it. It is also clear that by restraining the production of colored fruit many colonies would be rendered inconspicuous (although widely growing), thus accounting for the complaint so often heard with reference to dairy products taken from the refrigerators, that they turn green with mold very quickly.

SUMMARY OF DATA FROM COMPARATIVE CULTURE.

1. Species closely related in morphology and general appearance give closely similar reactions in culture under most conditions, but commonly show a few well-marked differences in special media or under special conditions.

2. Certain species will grow in media of widely differing composition; others require particular media for normal development.

3. Cane sugar in low concentrations is readily assimilated by every species studied.

4. Butterfat was attacked by nearly every species.

5. Lactose, galactose, levulose, and glycerin are assimilated by some species and not by others.

6. Potato starch produced normal growth in most species.

7. Very few species grew at 37° C.; i. e., very few species could be parasitic to warm-blooded animals. Few species were killed at 37° C., and species not killed grew normally when the medium was cooled to 20° C.

8. Incubation at low temperatures shows that growth in some species will begin at temperatures very close to the freezing point, but that only a few species will actually develop in cold-storage temperatures.

9. None of the species was found to grow in an atmosphere of carbon dioxide, but no species was killed by such atmosphere.

10. In most species failing to grow in a particular medium a change of concentration or the addition of a missing element will permit normal growth unless killed by osmotic pressure or definitely toxic agents.

KEYS TO CULTURAL IDENTIFICATION OF SPECIES.

Media: Prepare the following media—

1. 15 per cent gelatin^a in distilled water.

2. 15 per cent gelatin in distilled water plus 3 per cent cane sugar.

3. Either bean or potato decoction plus 1.5 per cent agar.

4. Bean or potato agar plus 3 per cent cane sugar.

Litmus solution may be added if desired when cultures are made.

Prepare Petri dishes with 10 c. c. of each of the media used and allow them to cool. Inoculate two or more Petri dishes of each medium with spores of the species under examination. Incubate at 20° C. (the temperature of the working laboratory is usually

^a In most of these studies the "gold-label" gelatin imported by Bausch & Lomb from Germany has been used. Culture of a species in a solution of gelatin in water has two uses in this paper—the detection of the production of enzymes capable of liquefying this medium, and the estimation of the ability of the species to grow in a medium free from carbohydrates. Neither of these tests seems to be vitiated by media made up at different times from materials of different origin. Such differences as are induced by the differences in the gelatin are quantitative, not qualitative.

satisfactory). Examine at intervals of three days or less, making observations with the naked eye from above and below, with the hand lens and with the compound microscope, using 16 mm. and 8 mm. or 6 mm. objectives to determine details of structure and fruit formation from growing colonies. A drop of litmus solution upon the margin of the colony will test acidity or alkalinity. Examine 1 and 2 for liquefaction, 2 and 4 for coremium and sclerotium formation. Sclerotium formation will be found to call for continued examination for at least two weeks.

Two separate keys are presented in the following pages: (1) A general key to all the forms discussed in this paper, based upon cultures in the media referred to above, and (2) a key to those species for which presence upon a particular substratum establishes a presumption of identity.

The species of most economic importance are found in key 2. Key 1, however faulty, is an endeavor to analyze the data from many series of comparative cultures in such a way as to simplify the identification of the forms included as far as possible. If the complexity of the data offered proves a barrier to identification it may be hoped that it will also deter those who fail to identify species clearly from using specific names in discussing work done with forms of this genus.

KEY 1.—ANALYSIS OF SPECIES IN CULTURES UPON GELATIN AND AGAR.

A. Species fruiting typically by coremia (vertical and definite).

a. Coremia long (3–15 mm.).

1. Conidial masses strictly terminal, olive green, fragrant. *P. claviforme*.
2. Upper third of coremia fertile, conidia green. *P. duclauxii*.

aa. Coremia small:

1. Coremia definite, densely crowded, colony orange below,
P. granulatum.
2. Coremiform character indicated in cultures by clustering of conidiophores, definite coremia only in old cultures, becoming large and definite upon apples. *P. expansum*.

AA. Species not (or rarely) producing coremia in culture.

B. Species constantly producing sclerotia or ascigerous masses.

b. Producing ascigerous masses, yellow or reddish. *P. luteum*.

bb. Sclerotia appearing as white masses in old cultures. *P. italicum*.^a

bbb. Sclerotia reddish or pink, globose or elliptical, 500 μ or less in diameter.

c. Conidial fructification a column:

1. Column dense, long, sclerotia partially buried in substratum,
P. No. 30.
2. Column formed of loose chains, sclerotia numerous, exposed,
P. No. 29.

cc. Conidial fructification of divergent chains:

1. Rapid liquefier, spores globose, 2.5–3 μ *P. No. 31*.
2. Slow liquefier, spores elliptical, 3.5–4 $\times$ 2.5–3 μ *P. No. 32*.

^aIn its earlier development *P. italicum* Wehmer will be usually thrown under the head *kk*, on account of its habit of growth.

BB. Sclerotia not (or rarely) produced (under special conditions).

(Use gelatin cultures (1) and (2), compare agar cultures).

C. Rapid liquefiers (abundant liquid in 5 to 12 days).

D. With definite, strong ammoniacal odor:

1. Yellowish brown-avellaneous spores rough.....*P. brevicaulis*.
2. White or cream, spores rough.....*P. brevicaulis*, var. *album*.
3. White or cream, spores smooth.....*P. brevicaulis*, var. *glabrum*.

DD. Without ammoniacal odor.

E. With yellow coloration of liquefied gelatin (*not of mycelium in reverse*).

1. Colonies small, conidiophores 100–150 μ in length.....*P. citrinum*.
2. Colonies broadly spreading, conidiophores 250–300 μ*P. chrysogenum*.

EE. Without yellow color in liquefied gelatin (or slight traces only).

e. Colonies white to pink or salmon.....*P. roseum*.

ee. Colonies some shade of green.

f. Colonies floccose, margin spreading by stolons.....*P. stoloniferum*.

ff. Colonies velvety-surface growth of fruiting hyphæ only.

g. Conidiophores very short (100–200 μ):

- 1.....*P.* No. 12.
- 2.....*P.* No. 37.

gg. Conidiophores longer (200–400 μ):

1. Conidiophores variously branched, reverse always colorless...*P.* No. 24.
2. Conidiophores each with a verticil of branches—each branch bearing a columnar fructification—reverse and medium darkened in sugar media.....*P. atramentosum*.

CC. Liquefaction of gelatin none or slower than 10–12 days, or only partial.

G. Colonies never green.

h. Colonies yellowish brown, spores elliptical.....*P. divaricatum*.

hh. Colonies white to lilac, slow liquefier, 14–16 days.....*P. lilacinum*.

hhh. Colonies floccose white or creamy:

1. Conidiophores long, typical penicillate branching,
P. camemberti, var. *rogeri*.
2. Conidial chains borne upon short branches of floccose hyphæ,
P. No. 33.

GG. Colonies some shade of green.

H. Surface with hyphæ definitely in ropes or trailing, bearing numerous conidiophores as short branches distinctly traceable to their origin in such hyphæ.

i. Colonies usually red below and reddening the substratum.

1. Fruiting areas dark green.....*P. funiculosum*.
2. Fruiting areas mixed yellow and green.....*P. pinophilum*.

ii. Colonies not producing red color:

1. Colonies gray rarely greenish, very loose floccose.....*P. intricatum*.
2. Colonies green, conidial chains in simple compact columns...*P.* No. 28.
3. Colonies gray to green, hyphæ scattered, creeping.....*P. decumbens*.

HH. Surface hyphæ not in well-defined ropes, nor trailing.

- j. Surface hyphæ woven floccose, course of hyphæ not traceable.
 - 1. Gray-green, long conidiophores, no odor.....*P. camemberti*.
 - 2. Gray-green, shorter conidiophores, strong odor.....*P. bisforme*.
- jj. Surface growth at margin simple conidiophores, in older parts both floccose hyphæ and conidiophores.
 - 1. Gray-greenish, branching of conidiophore rather loose, odor none or slight.....*P. No. 22*.
 - 2. Green, conidial fructifications rather compact, odor definite, "moldy".....*P. commune*.

N. B.—There are probably a number of races in this group.
- jjj. Fruiting surface velvety—of simple conidiophores or conidiophores borne so close to surface of substratum as to appear simple:
- k. Conidial mass a dense column of conidial chains.
 - 1. Column from a single verticil of basidia.....*P. spinulosum*.
 - 2. Column from a verticil of brachlets with verticillate cells and chains.....*P. rubrum*.
- kk. Elements of conidial fructification not in a column.
- l. Conidia smooth.
 - 1. Green, broadly spreading, ripe conidia globose, 4–5 μ*P. roqueforti*.
 - 2. Green, less spreading, conidia elliptical, medium, commonly purpled.....*P. purpurogenum*.
 - 3. Gray or olive green, conidia, 5–7 by 3–5 μ*P. digitatum*.
- ll. Conidia delicate rugulose.....*P. rugulosum*.

KEY 2.—SPECIES DETERMINABLE FROM SUBSTRATA.

(In these species the substratum establishes a presumption of identity.)

Cheese (Camembert and Brie):

- 1. Floccose, white unchangeable, no odor.....*P. camemberti* var. *rogeri*.
- 2. Floccose, white to gray-green, no odor.....*P. camemberti*.
- 3. Powdery, yellowish white, spores smooth, ammoniacal odor,
P. brevicaulis var. *glabrum*.
- 4. Powdery, yellowish white, spores tuberculate, ammoniacal odor,
P. brevicaulis var. *album*.
- 5. Forming yellowish-brown areas, spores rough, ammoniacal odor...*P. brevicaulis*.

Cheese (Roquefort):

- 1. Green streaks inside the cheese.....*P. roqueforti*.

Citrus fruits:

- 1. Colonies of mold, blue-green.....*P. italicum*.
- 2. Colonies of mold, olive-green.....*P. digitatum*=*olivaceum*.

Pomaceous fruits (apples, pears, etc.):

- 1. Blue-green colonies finally producing coremia.....*P. expansum*.

Polyporaceæ (Boleti, Polypori, etc.):

- 1. Colonies green (yellowish green) spreading by stolons.....*P. stoloniferum*.

Wood (pine):

- Producing orange to red stains in pine wood.....*P. pinophilum*.

TABULAR STATEMENTS.

TABLE 1.—*Tabulation of salient characters of species of Penicillium.*

[*Terms used.*—Colony characters: "Surface" growth is "strict" when conidiophores only rise above the substratum; "floccose" when conidiophores arise as branches of aerial hyphae; "mixed" when they arise both ways. "Margin" refers to the edge of the growing colony, which is usually either broadly spreading (broad) or restricted in habit (narrow). The lengths of the conidiophores are given in micromillimeters, and the limits given are those usual in peptone milk-sugar gelatin or in bean agar, observed within ten days. The conidial fructifications are designated as "loose" when the chains are more or less divergent, as "columns" when the chains are closely parallel or actually adherent into solid masses. In measurements of conidia the approximate limits of variation in diameter are given for globose conidia or the variations of the long and short axes of elliptical forms. In some cases both forms are noted.]

No.	Name.	Colony.		Length of conidiophore.			Conidial fructification.		Size of conidia.
		Surface.	Margin.	Minimum.	Average.	Maximum.	Form.	Length.	
				Microns.	Microns.	Microns.		Microns.	Microns.
1	<i>P. pinophilum</i>	Floccose.....	Broad.....	100	100	200	Loose.....	Up to 120	2 by 3-3.6
2	<i>P. brevicaulis</i> var. <i>glabrum</i>	Mixed.....	do.....	10	30	30	do.....	Up to 110	6.5-7.5 by 7.5-9
3	<i>P. brevicaulis</i> var. <i>album</i>	do.....	do.....	10	30	30	do.....	do.....	7-8 by 8-10
4	<i>P. camemberti</i>	do.....	do.....	15	40	40	do.....	do.....	9-10
5	<i>P. camemberti</i> var. <i>rogeri</i>	Floccose.....	do.....	300	800	800	do.....	do.....	4.5-5.5
6	<i>P. claviforme</i>	do.....	do.....	100	800	800	do.....	do.....	4.5-5.5
7	<i>P. claviforme</i>	do.....	do.....	100	800	800	do.....	do.....	4.5-5.5
8	<i>P. claviforme</i>	do.....	do.....	100	800	800	do.....	do.....	4.5-5.5
9	<i>P. granulosum</i>	Floccose.....	do.....	Coremia 5,000-15,000.	Coremia 5,000-15,000.	Coremia 5,000-15,000.	Columns.....	Up to 1,000-2,000	4.2-4.6 by 3-3.3
10	<i>P. granulosum</i>	do.....	do.....	Very short, mostly.	Very short, mostly.	Very short, mostly.	Loose.....	Up to 100	2.5-3 by 2
11	<i>P. italicum</i>	Strict.....	do.....	100	250	600	do.....	Up to 300	2.5-3 by 3-3.5, or 3
12	<i>P. italicum</i>	Floccose.....	do.....	20	30-60	100	do.....	Up to 80	2.3 by 3.5
13	<i>P. italicum</i>	Strict.....	Narrow.....	40	65	125	do.....	Up to 80	2.4 by 2.3
14	<i>P. expansum</i>	Mixed.....	Broad.....	100	100-200	300	Loose.....	40-80	3-3.5
15	<i>P. citrinum</i>	do.....	do.....	50	100-200	1,000	do.....	130-1,000	2 by 3.3, or 3-3.4
16	<i>P. digitatum</i>	do.....	Broad.....	30	150	100+	do.....	50-150	2-4.3
17	<i>P. purpurogenum</i>	do.....	Narrow (?).....	Variable with the media.	Variable with the media.	Variable with the media.	Loose.....	Up to 110	4.7 by 6-8
18	<i>P. roqueforti</i>	Strict.....	Broad.....	200	200	200	Loose columns.....	90-100	2-2.5 by 3.5-3.8
19	<i>P. roqueforti</i>	Floccose.....	do.....	45	125	125	Masses.....	Up to 140	4.5
20	<i>P. roqueforti</i>	do.....	do.....	15	10-30 or coremia.	30	Loose.....	Up to 140	5-7 by 3-5
21	<i>P. dauciaci</i>	Two forms.....	do.....	100	300	600	Loose.....	100-150	3.0-4 by 2-2.5
22	<i>P. rubrum</i>	Mixed.....	do.....	100	300	700	Loose.....	100-200	2.5 by 3.3
23	<i>P. rubrum</i>	Floccose.....	Broad.....	100	300	700	Loose.....	80-180	3-4
24	<i>P. commune</i>	do.....	do.....	100	300	700	do.....	Up to 200	3.3-4
25	<i>P. commune</i>	do.....	do.....	100	300	700	do.....	Up to 200	3-4
26	<i>P. chrysogenum</i>	do.....	Narrow (?).....	100 or less	300	100	Loose.....	Up to 170	28-3.4
27	<i>P. stoloniferum</i>	do.....	Broad.....	80	200	400	do.....	Up to 500	2-2.4 by 3-3.3, or 2-3
28	<i>P. stoloniferum</i>	Floccose.....	do.....	Very short or very long.	Very short or very long.	Very short or very long.	Loose columns.....	Up to 400	3-3.6 by 2.3-2.8
29	<i>P. stoloniferum</i>	do.....	do.....	250	300	500	Loose.....	Up to 500	2.5-3
30	<i>P. stoloniferum</i>	do.....	do.....	250	300	500	Loose.....	Up to 500	3.5-4 by 2-3
31	<i>P. stoloniferum</i>	do.....	do.....	250	300	500	Loose.....	Up to 500	3.5-4 by 2-3
32	<i>P. stoloniferum</i>	do.....	do.....	250	300	500	Loose.....	Up to 500	3.5-4 by 2-3
33	<i>P. stoloniferum</i>	Floccose.....	do.....	250	300	500	Loose.....	Up to 500	3.5-4 by 2-3

34	<i>P. disparfectum</i>	Strict	do.	100	100	200	do.	Up to 100	5-7 by 2.5-3
35	<i>P. decumbens</i>	Flaccose	Narrow	100	100	200	Columns	Up to 600	2.5-3
36		Strict	do.	240	300	400	do.	100-200	2.5-4 by 2.5-3
37		do.	do.	60-80	80	100	Loose	100-200	2.5-4.3
38	<i>P. sinuatum</i>	Two forms	Broad	20	80	100	do.	125-180	2.4 by 2.3
39	<i>P. biforme</i>	Flaccose	do.	150-300 or more	200	200	Columns	Up to 300+	2.2-3 by 3.6-4
40	<i>P. fructosum</i>	Mixed	do.	100	200	200	Loose	Up to 150	2.4-3 by 2.5-3
41	<i>P. spinulosum</i>	Strict	Narrow	30	50	50	Loosely columnar	50-140	2.5-3
42	<i>P. spinosum</i>	Flaccose	Broad	30	50	50	do.	Up to 300	3
43	<i>P. fructuosum</i>	Strict (?)	Narrow	30	50	50	do.	Up to 300	3

TABLE 2.—*Gelatin and color reactions.*

Species.		15 per cent gelatin in water.		Color. ^a		
No.	Name.	Liquefaction.	Litmus.	In conidial mass.	In medium.	Reverse of colony.
1	<i>pinophilum</i>	Very slow, 6 weeks.	Acid.....	Green.....	Yellow to red..	Red.
2	<i>brevicaule</i>	Rapid, 5 to 6 days.	Alkaline.....	Drab to chocolate.	None.....	None.
3	var. <i>glabrum</i>	do.....	do.....	White or cream	do.....	Do.
4	var. <i>album</i>	do.....	do.....	do.....	do.....	Do.
5	<i>camemberti</i>	None or very slow.	do.....	Gray-green.....	do.....	Cream.
6	var. <i>rogeri</i>	do.....	do.....	White.....	do.....	Do.
7	<i>claviforme</i>	Slow.....	Neutral.....	Olive.....	Brownish.....	Brownish.
8	<i>lilacinum</i>	14 to 16 days.....	Alkaline.....	White or lilac.....	None.....	None.
9	<i>granulatum</i>	None.....	do.....	Yellowish green.	Yellow to orange.	Yellow to orange.
10	<i>italicum</i>	None or slow.....	Faint alkaline.	Bluish green.....	None.....	Brownish areas.
11	<i>luteum</i>	None.....	Acid.....	Green, small areas.	None or ?.....	Yellow to orange.
12		6 days.....	Alkaline.....	Pale blue-green.	None.....	None.
13		None in 15 days.	do.....	Deep green.....	do.....	Do.
14	<i>expansum</i>	Partial, 14 days.	do.....	Green to gray-green.	do.....	None or brownish.
15	<i>citrinum</i>	Rapid, 4 to 6 days.	do.....	Green.....	Lemon yellow.	None or yellowish.
16	<i>digitatum</i>	None in 15 days.		Olive.....	None.....	Brownish.
17	<i>purpuregenum</i>	do.....		Green.....	Red or none.....	Yellow to red or none.
18	<i>roqueforti</i>	do.....	Alkaline.....	do.....	None.....	None.
19	<i>roseum</i>	Rapid, 8 days.....	do.....	White or salmon.	do.....	White or cream.
20	<i>duclauxi</i>	None in 15 days.	See color.....	Olive or green.	(Yellow, acid..)	Yellow, acid.
21	<i>rubrum</i>	(?).....	(?).....	Green or various.	(Red, alkaline..)	Red, alkaline.
22		Partial, 15 to 20 days.	Slow alkaline.	Gray-green.....	None.....	White or cream.
23	<i>commune</i>	do.....	Alkaline.....	Green.....	do.....	Do.
24		Rapid 8 to 10 days	do.....	Deep green.....	do.....	Do.
25	cf. 26.....	do.....	do.....	Green.....	Yellow in certain media.	White or yellowish.
26	<i>chrysogenum</i>	do.....	do.....	do.....	None or golden	None or yellowish.
27	<i>stoloniferum</i>	Rapid, 6 to 8 days.	do.....	Yellowish green.	None or ?.....	Cream or yellowish.
28		Very slow, 20+ days.	Acid.....	Gray-green to green.	Yellow! violet!	None or yellowish.
29		Rapid.....	Alkaline.....	White, some green.	None or slight.	Do.
30		Very slow.....	Acid.....	Green.....	None.....	None.
31		Rapid.....	Alkaline.....	Gray or greenish.	None or yellowish.	None or yellowish.
32		Partial, slow, 15+ days.	Neutral?.....	Gray-green.....	None or reddish.	Yellowish to salmon.
33		None.....	Acid.....	White or cream.	None.....	Cream.
34	<i>divaricatum</i>	do.....	do.....	Yellowish brown.	do.....	None.
35	cf. 26.....	Rapid.....	Alkaline.....	Gray or green.	None.....	White.
36	<i>decumbens</i>	None or ?.....	Neutral?.....	Gray to blue-green.	do.....	Do.
37		Rapid, 6 to 7 days.	Alkaline.....	do.....	do.....	Do.
38	<i>atramentosum</i>	Rapid, 6 to 10 days	do.....	Deep green.....	None or brown	None or brown.
39	<i>biforme</i>	None in 15 days.	do.....	Gray-green.....	None.....	Cream.
40	do.....	do.....	do.....	Green.....	do.....	Do.
41	= 19.....	Rapid.....	do.....	(?).....	(?).....	(?)
42	<i>funiculosum</i>	None.....	Acid.....	Green.....	Red or none.....	Red or none.
43		None or ?.....	Neutral?.....	Light olive.....	Red.....	Orange to red.
44	cf. 26.....	Rapid.....	Alkaline.....	(?).....	(?).....	(?)
45	<i>spinulosum</i>	Partial, slow	Acid.....	Green.....	None.....	Cream or violet.
46	<i>rugulosum</i>	None or ?.....	Neutral?.....	do.....	None or yellowish.	Yellow to orange red.
47		Rapid.....	Alkaline.....	(?).....	(?).....	(?)
49	<i>intricatum</i>	None.....	do.....	Gray to greenish.	None.....	White or sulphur.
51		None or ? slow	do.....	Yellowish green.	do.....	Uncolored.
54		Partial and slow.	do.....	(?).....	(?).....	(?)
56	= 38.....	Rapid.....	do.....	(?).....	(?).....	(?)

^a "None" means the absence of a definite color, varying from hyaline to cream at times.

TABLE 3.—Comparative cultures.

No.	Cohn's solution.	Raulin's fluid.	Milk + CaCl ₂ , curdling.	Milk.		Natural substratum.	Data at 37° C.
				Rate of digestion.	Coloration.		
1	Germinated		Slow, 15 days.	Very slow	Red	Wood staining.	Grew best.
2	Slow, but typical.	Germinated.	10 days.	Rapid	None	Soft cheese	Grew well.
3	Germinated only.	do.	13 days.	do.	do.	do.	No growth.
4	do.	do.	8 days.	do.	do.	do.	Do.
5	Fair, white	Very heavy.	9 days.	Slow	do.	Camembert cheese.	Do.
6	No growth.	do.	do.	do.	do.	do.	Do.
7	Slight.	Typical.	do.	do.	Reddish brown.	do.	Do.
8	Weak, typical.		None.	do.	None.		Best growth.
9	Germinated only.	Typical.	do.	do.	Slowly red.		No growth.
10	do.	do.	10 days.	do.	Yellowish.	Citrus fruits.	Killed.
11	do.	Slow, yellow.	Very slow growth in milk.				Best growth.
12	Slight.	Slow, typical.	7 days.	Rapid	None.		Killed.
13	Small growth	Good.	10 days.	Slow	do.		No growth.
14	Weak.	Typical.	8 days.	do.	Yellowish brown.	Apples.	Do.
15	Good, no yellow.	Good, some yellow.	do.	Rapid	Slight yellow.		Slight growth.
16	Germinated.	Some growth	None.	poor growth in milk.		Oranges.	Killed.
17	do.	Slow growth.	13 days.	Slow	Slight red.		Grew well.
18	Slight growth	Richly typical.	8 days.	Rapid	None, or evanescent greenish.	Roquefort cheese.	None.
19	do.		Rapid	do.	None.		Killed.
20	Germinated.	Typical.	9 days.	Slow	Yellow to red.		No growth.
21			13 days.	do.	None.		
22	Fair growth.		8 days.	do.	do.		Do.
23	Small colonies.	Typical.	7 days.	do.	do.		Do.
24	Slowly typical.	Slow, typical	8 days.	Rapid	do.		Do.
25	Weak.		do.	do.	Pale yellow.		Slow growth.
26	Not typical.	Typical.	7 days.	do.	Golden yellow.		Good growth.
27	Typical.	Slowly typical.	do.	do.	Slight, if any.	Fleshy fungi.	Killed.
28	Weak growth	Typical.	8 to 9 days.	Slow	Pale yellow.		Do.
29		do.	9 days.	do.	None.		
30	Germinated		do.	do.	do.		No growth.
31	Slowly typical.	Not typical.	do.	Rapid	do.		Do.
32	Small colonies.	Good growth	Very slow.	Slow	do.		Grew best.
33	No growth.	Typical.	From top down.	Slight.	do.		Killed.
34	Germinated.	do.	Slow.	Little or none.	do.		Grew best.
35			9 days.	Medium rapid.	Yellow.		
36	Germinated.	Richly typical.	Very slow, 4 weeks.	Little or none.	None.		Slight growth.
37	Weak growth	Typical.		Fairly rapid.	do.		Killed.
38	Germinated.	Weak growth	9 days.	Rapid.	Reddish brown.		No growth.
39	Half typical.		8 days.	Rather slow.	None.		Do.
40	do.		do.	do.	do.		Do.
41			9 days.	do.	do.		
42	Germinated.	Typical.	Very slow.	Little	Red where touched.		Grew well.
43	No growth.		9 days.	Slow, slight.	Not colored.		Do.
44			7 days.	Rapid.	Golden yellow.		
45	Germinated.	Typical.	Very slow.	Slow.	None.		Grew best.
46			9 days.	Slight.	do.		No growth.
47			8 days.	Slow.	do.		
48	Weak growth		Slow, 3 weeks.	Very slow.	do.		Grew best.
51	Small colonies.		None, 4 weeks.	do.	do.		No growth.
54			11 days.	Slow.	do.		
56							

TABLE 4.—*Comparative cultures in synthetic fluid (Dox's).*

No.	Carbon supplied as—					0.9 per cent lactic acid.
	Cane sugar.			Lactose.		
	1 to 20 per cent.	60 per cent.	75 per cent.	Growth.	Litmus.	
1	Typical	No growth		Not typical	No effect	Germinated.
2	Slowly typical	Germinated only.	Slight growth	Slow, typical	do	
3	do	Slight.	do	do	do	Typical, alkaline.
4	do	do	do	do	do	
5	Typical	Slowly typical.	Slow	Typical	Acid	Do.
6	do	do	Slight	do	do	
7	do	No coremia but acid.		Very weak growth.	No effect	Germinated.
8	do	Slight.	No growth	Germinated only.	do	
9	do	Not typical	Germinated	Slow, weak	do	Not typical.
10	do	Slow, typical	No growth	Slight	Acid	
11	do	No growth	Germinated	do	No effect	Do.
12	do	Good, acid	Slow	Weak, typical	do	
13	do	do	do	Slow, typical	do	Good.
14	do	do	Not typical	do	do	
15	do	Weak, no yellow.	Weak, half typical.	Slow growth	do	Slight.
16	Not typical	Germinated only.	No growth	Germinated	do	
17	Typical	do	do	do	do	Slow.
18	do	Not typical	Weak	Weak	do	
19	do	No growth	No growth	Slight	do	Weak.
20	Not typical	Germinated only.	do	Germinated	do	
21			do	do	do	Good, acid.
22	Typical	Slowly good	Very poor	Rich growth	Acid	
23	do	do		do	do	Good.
24	do	Weak	Germinated, small.	Small growth	No effect	
25	do	Good	Good	Typical	Acid	Do.
26	do	do	do	Slowly typical	do	
27	do	do	do	Weak	No effect	Typical.
28	do	do	Fair	Typical	Acid	
29	do	do	No growth	Slight	No effect	Small colonies.
30	Typical	Green, no sclerotia.	No growth	Slowly typical	Acid	
31	Not typical	do	Slight	Slight	No effect	Not typical.
32	Typical	Weak	No growth	do	do	
33	do	Germinated only.	do	do	do	Good.
34	do	Slight	do	do	do	
35	do	Good	Good growth	Typical	Acid	Fair, not typical.
36	do	Very weak	Pinhead colonies.	Slight	No effect	
37	do	Slowly typical.	Half typical	Slowly growing	do	Do.
38	do	do	Very weak	Slowly typical	do	
39	do	do	Slight	Typical	Acid	Small colonies.
40	do	do	do	do	do	
41	Slowly typical	Weak	No growth	Slight	No effect	No growth.
42	Typical	do	do	do	do	
43	do	do	do	do	do	Typical, alkaline.
44	do	Slowly typical.	Good growth	Typical	Acid	
45	do	do	Pinhead colonies.	Slight	No effect	Little growth.
46	do	Fair, not typical.	Germinated	do	do	
47	do	Typical	Weak	Good	Acid	Do.
48	do	Weak	No growth	do	No effect	
49	do	Very small colonies.	Pinhead colonies.	Germinated	do	Small colonies.
50	do	Typical	Good	Slight	do	
51	do	Slowly typical.	Weak	Slow, typical	do	No growth.
52	do	do	do	do	do	
53	do	do	do	do	do	Typical, alkaline.
54	do	do	do	do	do	
55	do	do	do	do	do	Do.
56	do	do	do	do	do	

TABLE 4.—*Comparative cultures in synthetic fluid (Dox's)*—Continued.

No.	Carbon supplied as—				
	2.5 per cent levulose.	3 per cent galactose.	3 per cent glycerin.	Butterfat.	
				Growth.	Color in medium.
1	Good, acid.....	Fair, acid.....	Slight.....	Typical, slow.....	Yellow to red.
2	Fair, alkaline.....	Fair, alkaline.....	Germinated only.....	Slow.....	
3	do.....	do.....	do.....	Fair.....	
4	do.....	do.....	Slight colony.....	do.....	None.
5	Typical.....	Typical.....	Slow, typical.....	Slow, typical.....	
6	do.....	do.....	do.....	do.....	
7	Weak.....	Good, acid.....	No coremia.....	do.....	Do.
8	Slow.....	Slow, alkaline.....	Slight.....	do.....	Tinged.
9	Slow, small.....	Typical.....	do.....	do.....	Tinged brownish.
10	Not normal.....	Fair, acid.....	No growth.....	do.....	Yellowish.
11	Slight.....	Good, acid.....	Germinate.....	Slight.....	None.
12	Good, alkaline.....	Good, alkaline.....	Slow, not typical.....	Weak.....	Do.
13	do.....	do.....	Good.....	Good.....	Do.
14	do.....	do.....	Slow.....	do.....	Do.
15	Small colonies.....	do.....	Small colonies.....	do.....	Lemon yellow.
16	Germinated.....	Slight.....	Germinated.....	None.....	Slowly red.
17	Small colonies.....	Small, acid.....	No growth.....	Slow.....	
18	Weak.....	Good colonies.....	Weak.....	Good.....	
19	Slight.....	do.....	Slight.....	Slowly typical.....	Do.
20	Weak.....	Weak.....	do.....	do.....	Yellowish.
21	do.....	do.....	do.....	do.....	
22	Good.....	Typical.....	Typical.....	Typical.....	None.
23	do.....	do.....	do.....	do.....	Do.
24	Small.....	do.....	Weak.....	do.....	Do.
25	Typical.....	do.....	do.....	do.....	
26	do.....	do.....	do.....	do.....	
27	Typical.....	Typical.....	Fair colonies.....	do.....	
28	Slow, typical.....	do.....	do.....	Typical.....	Reddish brown.
29	do.....	do.....	do.....	Slow.....	None.
30	Fair.....	Typical.....	Very weak.....	Very slow.....	Do.
31	Typical.....	do.....	do.....	Very slow, typical.....	Do.
32	Slowly typical.....	do.....	Very small colonies.....	do.....	Do.
33	Small growth.....	do.....	Slowly typical.....	Slowly typical.....	Slightly rusty.
34	do.....	Slow.....	Germinated.....	Weak.....	None.
35	do.....	do.....	do.....	do.....	
36	Small colonies.....	Small growth.....	Germinated.....	Weak.....	Do.
37	do.....	Typical.....	Slight.....	Slow.....	Do.
38	Slowly typical.....	do.....	Germinated.....	Typical.....	Do.
39	Typical.....	do.....	Typical.....	do.....	Do.
40	do.....	do.....	do.....	do.....	Do.
41	do.....	do.....	do.....	do.....	
42	Little growth.....	Little growth.....	Germinated.....	Coremiform colonies.....	Do.
43	do.....	do.....	do.....	Slow, typical.....	Yellowish.
44	do.....	do.....	do.....	do.....	
45	Typical.....	do.....	Half typical.....	Typical.....	None.
46	Good.....	Good.....	Slow.....	Slowly typical.....	Slight yellowish.
47	do.....	do.....	do.....	do.....	
49	Typical.....	Typical.....	Slow.....	Fair.....	None.
51	Half typical.....	do.....	Slowly typical.....	Small, typical.....	Do.

TABLE 5.—*Decimal summary of comparative cultures in synthetic fluid (Dox's).*

Species.		Carbon supplied as—												
		Cane sugar.				Lactose.		Levulose, growth.	Glycerin, growth.	Galactose, growth.	Lactic acid, growth.	Raulin's fluid, growth.	Cohn's solution, growth.	Characteristic odor.
		Up to 20 per cent.		60 per cent, growth.	75 per cent, growth.	Growth.	Acid.							
No.	Name.	Growth.	Acid.											
1	<i>pinophilum</i>	1.0	1.0	0.1	0.0	0.0	0	1.0	0.2	0.8			0.1	0
2	<i>brevicaule</i>	-1.0	0.0	0.1	0.1	-1.0	0	0.8	0.1	0.8	0.1	0.1	-1.0	0
3	var. <i>glabrum</i>	-1.0	0.0	0.1	0.1	-1.0	0		0.1			0.1	0.1	1
4	var. <i>album</i>	-1.0	0.0	0.1	0.1	-1.0	0		0.3			0.1	0.1	1
5	<i>camemberti</i>	1.0	1.0	-1.0	0.5	1.0	1	1.0	-1.0	1.0	1.0	+1.0	0.8	0
6	var. <i>rogeri</i>	1.0	1.0	-1.0	0.5	1.0	1	1.0	-1.0	1.0	1.0	+1.0	0.0	0
7	<i>claviforme</i>	1.0	1.0	0.2	0.2	0.2	0	0.4	0.3	0.9	0.1	1.0	0.0	1
8	<i>lilacinum</i>	1.0	(?)	0.1	0.0	0.1	0	0.4	0.2	0.8	0.3	1.0	-0.7	0
9	<i>granulatum</i>	1.0	1.0	0.3	0.1	0.5	0	0.5	0.4	1.0	0.6	1.0	0.1	0
10	<i>italicum</i>	1.0	0.0	-1.0	0.0	0.3	?	0.5	0.3	0.9	0.3	1.0	0.1	1
11	<i>luteum</i>	1.0	0.0	0.1	0.1	0.2	0	0.2	0.1	1.0	0.3	-0.7	0.1	0
12		1.0	1.0	0.1	0.6	0.7	0	1.0	0.4	1.0	0.4	-1.0	0.2	0
13		1.0	1.0	1.0	0.8	-1.0	0	1.0	-1.0	1.0	0.8	1.0	0.4	1
14	<i>expansum</i>	1.0	1.0	1.0	0.4	-1.0	(?)	1.0	-1.0	1.0		1.0	0.5	1
15	<i>citrinum</i>	1.0	1.0	0.5	0.5	-0.6	0	0.6	0.6	1.0	0.4	1.0	0.7	0
16	<i>digitatum</i>	(?)	(?)	0.1	0.0	0.1	(?)	0.1	0.1	0.4	0.1	0.4	0.1	1
17	<i>purpurogenum</i>	1.0	0.0	0.1	0.0	0.1	0	0.3	0.0	0.4	0.6	0.6	0.1	0
18	<i>roqueforti</i>	1.0	0.0	0.3	0.2	0.5	0	0.5	0.4	0.8	0.4	1.0	0.2	0
19	<i>roseum</i>	-1.0	0.0	0.0	0.0	0.2	0	0.2	0.2				0.2	0
20	<i>duclauxi</i>	1.0	(?)	0.1	0.0	0.1	0	0.2	0.2		0.3	0.8	1.0	(?)
21	<i>rubrum</i>	1.0	1.0	-1.0	0.3	-1.0	1	1.0	-1.0	1.0			0.8	(?)
22		1.0	1.0	-1.0	0.6	-1.0	1	1.0	-1.0	1.0	1.0	1.0	0.5	1
23	<i>commune</i>	1.0	1.0	0.4	0.3	0.6	0	0.6	0.5	1.0	1.0	-1.0	-1.0	0
24		1.0	1.0	0.7	0.7	1.0	1	1.0	0.7	1.0	1.0		0.5	(?)
25		1.0	1.0	0.7	0.7	-1.0	1		0.7			-1.0	0.5	0
26	<i>chrysogenum</i>	1.0	1.0	1.0	-1.0	0.6	0	1.0	0.9	1.0	0.5	-1.0	1.0	0
27	<i>stoloniferum</i>	1.0	1.0	1.0	-1.0	1.0	1	-1.0	0.9		0.5	1.0	0.6	(?)
28		1.0	1.0	0.0	0.2							1.0		0
29		1.0	1.0	0.4	0.2	-1.0	1	1.0	0.2	1.0	0.4		0.1	0
30		1.0	1.0	0.5	0.2	0.2	0	1.0	0.2	1.0	0.0	0.5	-1.0	0
31		1.0	1.0	0.3	0.0	0.2	0	-1.0	0.3	1.0	1.0	0.8	0.6	0
32		1.0	(?)	0.1	0.0	0.2	(?)	0.3	-1.0	1.0	1.0	1.0	0.0	0
33		1.0	1.0	0.1	0.0	0.2	0	0.3	0.1	0.4	0.7	1.0	0.1	0
34	<i>divaricatum</i>	1.0	1.0	0.1	0.0	0.7	1							0
35	cf. 26.....	1.0	1.0	0.2	0.5	0.2	0	0.3	0.1	0.4	0.7	1.0	0.1	1
36	<i>decumbens</i>	1.0	1.0	0.5	0.8	0.5	0	0.5	0.5	1.0	0.7	1.0	0.3	0
37		1.0	1.0	0.7	0.3	0.9	0	0.9	0.1	1.0	0.0	0.5	0.1	0
38	<i>atramentosum</i>	1.0	1.0	0.4	1.0	1	1	-1.0	-1.0	1.0	1.0		0.7	1
39	<i>biforme</i>	1.0	1.0	-0.9	0.4	1.0	1	1.0	0.8	1.0			0.7	1
40		1.0	1.0	-0.9	0.0	0.2	0	0.2	0.1	0.3	0.3	1.0	0.1	0
42	<i>funiculosum</i>	1.0	1.0	0.1	0.0	0.2	0	0.2	0.1	0.3	0.3		0.0	0
43	cf. 17.....	1.0	1.0	0.1	0.0	0.2	0	0.2	0.1	0.3	0.3			0
44	cf. 26.....	1.0	1.0	0.7	0.8	1.0	1		0.5					0
45	<i>spinulosum</i>	1.0	1.0	-0.8	0.5	0.2	0	1.0	0.6			1.0	0.1	0
46	<i>rugulosum</i>	1.0	1.0	-0.6	0.1	0.2	0	1.0	-1.0	0.9				0
49	<i>intricatum</i>	1.0	1.0	0.3	0.0	1.0	0	1.0	-1.0	1.0			0.3	0
51		1.0	1.0	-1.0	0.8	0.2	0		0.1	1.0				0

Explanation of decimals: 1.0 denotes normal development, or present; -1.0, slowly typical; 0.1, germination of spores only; decimals from 0.1 to 1.0 denote estimated amount of development between mere germination and typical development; +1.0 denotes very rich growth.

TABLE 6.—Incubation experiments.

No.	Species.	20° C.	Six days at 37° C.	Ice thermostat.															
				Compartment 1, 0.5–2° C.				Compartment 2, 3.2–6° C., av. 4° C.				Compartment 3, 6–10° C., av. 7°+ C.				Compartment 5, 7–10.5° C., av. 8.7° C.			
				Growth, periods in days.				Growth, periods in days.				Growth, periods in days.				Growth, periods in days.			
				7.	15.	23.	29.	7.	15.	23.	29.	7.	15.	23.	29.	7.	15.	23.	29.
1	<i>pinophilum</i>	1.0	+1.0	0.0	0.1	0.1	0.1	0.1	0.1?	0.1?	0.1?	0.1	0.1?	0.1?	0.1?	0.1	0.3	0.5	0.6
2	<i>brevicaule</i>	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7
3	var. <i>glabrum</i>	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.4	0.4	0.3	0.4	0.5	0.7
4	var. <i>album</i>	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	<i>camemberti</i>	1.0	0.0	0.0	0.2	0.5	0.6	0.3	0.5	0.5	0.6	0.5	0.6	0.7	0.9	0.6	0.6	1.0	1.0
6	var. <i>rogeri</i>	1.0	0.0	0.0	0.0	0.1	0.3	0.5	0.1	0.3	0.4	0.5	0.3	0.5	0.6	0.8	0.4	0.8	1.0
7	<i>claviforme</i>	1.0	0.0	0.0	0.2	0.3	0.4	0.6	0.1	0.3	0.4	0.6	0.3	0.6	0.8	0.9	0.6	0.6	0.8
8	<i>lilacinum</i>	1.0	+1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1?	0.1?	0.1	0.0	0.3	0.5
9	<i>granulatum</i>	1.0	0.0	0.0	0.2	0.3	0.4	0.6	0.3	0.5	0.6	0.8	0.3	0.5	0.6	0.8	0.6	0.8	1.0
10	<i>italicum</i>	1.0	0.0	1.0	0.1	0.8	0.9	0.9	0.3	0.7	0.8	0.9	0.5	0.8	0.9	1.0?	0.8	1.0	1.0
11	<i>luteum</i>	1.0	+1.0	0.0	0.0	0.0	0.1?	0.1?	0.0	0.0	0.0	0.0	0.0	0.1?	0.1?	0.1?	0.1	0.3	0.5
12		1.0	0.0	1.0	0.0	0.0	0.0	0.1?	0.0	0.0	0.1?	0.1?	0.0	0.0	0.0	0.0	0.2	0.2	0.2
13		1.0	0.0	0.0	0.2	0.5	0.6	0.8	0.3	0.5	0.7	0.9	0.4	0.7	0.9	1.0	0.6	1.0	1.0
14	var. <i>expansum</i>	1.0	0.0	0.0	0.1	0.1	0.3	0.4	0.2	0.2	0.3	0.4	0.1	0.4	0.5	0.6	0.6	0.9	1.0
15	<i>citrinum</i>	1.0	0.3	0.0	0.1	0.0	0.0	0.1?	0.1	0.1?	0.1?	0.1?	0.1	0.1	0.1	0.1	0.2	0.3	0.3
16	<i>digitatum</i>	1.0	0.0	1.0	0.1	0.1	0.3	0.4	0.2	0.2	0.3	0.4	0.1	0.4	0.5	0.6	0.5	0.7	0.8
17	<i>purpurogenum</i>	1.0	1.0	0.0	0.0	0.1?	0.1?	0.1?	0.1	0.1	0.1	0.1	0.0	0.1?	0.1?	0.1?	0.3	0.1	0.1
18	<i>roquefortii</i>	1.0	0.0	0.0	0.2	0.3	0.7	0.8	6.25	0.5	0.7	0.9	0.4	0.7	1.0	1.0	0.6	1.0	1.0
19	<i>roseum</i>	1.0	0.0	1.0	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1?	0.0	0.0	0.0	0.1	0.5	0.6
20	<i>duclauxi</i>	1.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1?	0.1?	0.1	0.1	0.3	0.4
21	<i>rubrum</i>	1.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22		1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23	<i>commune</i>	1.0	0.0	0.0	0.3	0.6	0.6	0.8	0.4	0.6	0.7	0.9	0.6	0.9	1.0	1.0	0.6	0.8	1.0
24		1.0	0.0	0.0	0.1	0.3	0.5	0.7	0.2	0.5	0.7	0.9	0.4	0.7	1.0	1.0	0.6	1.0	1.0
25	=26	1.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
26	<i>chrysogenum</i>	1.0	1.0	0.0	0.0	0.1	0.3	0.6	0.4	0.5	0.6	0.8	0.4	0.6	0.8	1.0	0.6	1.0	1.0
27	<i>stoloniferum</i>	1.0	0.0	1.0	0.2	0.1	0.1	0.3?	0.4	0.4	0.4	0.5	0.5	0.5	0.5	0.6	0.6	0.9	1.0
28		1.0	0.0	1.0	0.0	0.0	0.1	0.1	0.0	0.1?	0.1	0.3	0.1	0.3	0.4	0.6	0.4	0.6	0.8
29		1.0		0.0	0.0	0.1	0.3	0.3	0.0	0.1	0.2	0.6	0.1	0.5	0.5	0.6	0.4	0.7	0.8
30		1.0	0.0	0.0	0.0	0.1	0.4	0.5	0.0	0.0?	(?)	0.0	0.0	0.4	0.7	0.8	0.0	0.7?	(?)
31		1.0	0.0	0.0	0.2	0.5	0.6	0.6	0.4	0.5	0.7	0.8	0.5	0.8	1.0	1.0	0.6	0.7	0.8
32		1.0	+1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
33		1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
34	<i>divaricatum</i>	1.0	+1.0	0.0	0.0	0.0	0.0	0.0	0.1?	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.2	0.3
35	cf. 26	1.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
36	<i>decumbens</i>	1.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.2	0.1	0.3	0.4	0.4	0.2	0.7	0.7
37		1.0	0.0	1.0	0.1	0.1?	0.1?	0.1?	0.1	0.1?	0.1?	0.1?	0.1	0.1	0.1	0.1	0.1	0.3	0.3
38	<i>atramentosum</i>	1.0	0.0	0.0	0.1	0.1	0.3	0.5	0.1	0.1	0.4	0.7	0.2	1.0	1.0	1.0	0.8	0.9	1.0
39	<i>biforme</i>	1.0	0.0	0.0	0.4	0.4	0.7	0.9	0.4	0.2	0.5	0.8	0.5	0.6	0.7	0.9	0.6	0.9	1.0
40	cf. 23	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
41	=19	1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
42	<i>funiculosum</i>	1.0	1.0	0.0	0.0	0.0	0.0	0.1?	0.0	0.0	0.1	0.1	0.0	0.3	0.5	0.7	0.1	0.7	0.8
43	cf. 17	1.0	1.0	0.0	0.0	0.0	0.0	0.1?	0.1	0.0	0.1?	0.1?	0.1				0.1	0.3	0.4
44	cf. 26	1.0	-0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
45	<i>spinulosum</i>	1.0	+1.0	0.0	0.0	0.1?	0.1	0.3	0.1	0.1	0.2	0.3	0.4	0.5	0.7	0.7	0.6	0.7	1.0
46	<i>rugulosum</i>	1.0	0.0	0.0	0.2	0.1	0.1	0.2	0.2	0.1	0.2	0.3	0.4	0.5	0.5	0.6	0.5	0.7	0.8
47		1.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
48	<i>intricatum</i>	1.0	+1.0	0.0	0.0	0.0	0.0	0.1?	0.0	0.0	0.0	0.0	0.1	0.3	0.3	0.3	0.1	0.3	0.6
49		1.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.3	0.4	0.4	(?)	0.7?	0.8?
51		1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
52		1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
56	=38	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Explanation of decimals: 0.1 denotes germination of conidia only; decimals up to 0.7, growth without the formation of colored conidial areas; 0.7 to 1.0, colonies with colored conidia; 1.0 denotes typical colony. +1.0 denotes growth more rapid at 37° than at 20° C.

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INDEX OF SPECIES.

<i>Penicillium</i> —	Page.
<i>album</i> Epstein.....	50
<i>atramentosum</i>	65
<i>aureum</i> Corda, emended Hedgcock.....	37
<i>brevicaule</i> Saccardo.....	45
<i>brevicaule</i> Saccardo, var. <i>album</i> n. var.....	47
<i>brevicaule</i> Saccardo, var. <i>glabrum</i> n. var.....	48
<i>camemberti</i> Thom.....	50
<i>camemberti</i> Thom, var. <i>rogeri</i> n. var.....	52
<i>candidum</i> Link.....	52
<i>chrysogenum</i> n. sp.....	58
<i>citrinum</i> n. sp.....	61
<i>claviforme</i> Bainier.....	43
<i>commune</i> n. sp.....	56
<i>decumbens</i> n. sp.....	71
<i>digitatum</i> Saccardo.....	31
<i>divaricatum</i> n. sp.....	72
<i>duclauxi</i> Delacroix.....	42
<i>epsteinii</i> Lindau.....	50
<i>expansum</i> Link, emended.....	27
<i>funiculosum</i> n. sp.....	69
<i>glaucum</i> Link.....	26
<i>granulatum</i> Bainier.....	44
<i>intricatum</i> n. sp.....	75
<i>italicum</i> Wehmer.....	29
<i>lilacinum</i> n. sp.....	73
<i>luteum</i> Zukal.....	39
<i>olivaceum</i> Wehmer.....	31
<i>pinophilum</i> Hedgcock, n. nov.....	37
<i>purpurogenum</i> O. Stoll.....	36
<i>roquesforti</i> Thom.....	34
<i>roseum</i> Link (?).....	49
<i>rubrum</i> O. Stoll.....	39
<i>rugulosum</i> n. sp.....	60
<i>spinulosum</i> n. sp.....	76
<i>stoloniferum</i> n. sp.....	68

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U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY.—BULLETIN 119.
A. D. MELVIN, CHIEF OF BUREAU.

STUDIES ON BLOOD AND BLOOD PARASITES.

I. OBSERVATIONS ON MAMMALIAN BLOOD WITH DARK-FIELD
ILLUMINATION.

II. THE PRIORITY OF CRYPTOBIA LEIDY, 1846, OVER TRYPANO-
PLASMA LAVERAN AND MFSNIL, 1901.

III. TRYPANOSOMA AMERICANUM N. SP., A TRYPANOSOME WHICH
APPEARS IN CULTURES MADE FROM THE BLOOD OF AMERICAN
CATTLE. (*Preliminary Notice.*)

BY

HOWARD CRAWLEY,
Expert in Protozoology, Zoological Division.



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1909.

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LETTER OF TRANSMITTAL.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY,
Washington, D. C., August 26, 1909.

SIR: I have the honor to transmit herewith, and to recommend for publication as a bulletin of this Bureau, three papers concerning studies on blood and blood parasites, by Mr. Howard Crawley, expert in protozoology in the Zoological Division of this Bureau.

Respectfully,

A. M. FARRINGTON,
Acting Chief of Bureau.

Hon. JAMES WILSON,
Secretary of Agriculture.

CONTENTS.

	Page.
I. Observations on mammalian blood with dark-field illumination.....	5
II. The priority of <i>Cryptobia</i> Leidy, 1846, over <i>Trypanoplasma</i> Laveran and Mesnil, 1901.....	16
III. <i>Trypanosoma americanum</i> n. sp., a trypanosome which appears in cultures made from the blood of American cattle. (Preliminary notice.).....	21

ILLUSTRATIONS.

	Page.
Fig. 1. Beaded threads in blood.....	10
2. <i>Cryptobia heliciis</i> Leidy.....	18
3. <i>Trypanosoma americanum</i> n. sp.....	28

STUDIES ON BLOOD AND BLOOD PARASITES.

I. OBSERVATIONS ON MAMMALIAN BLOOD WITH DARK-FIELD ILLUMINATION.

INTRODUCTION.

In the course of a study made on mammalian blood, having for its object the cultivation in vitro of *Piroplasma bigeminum*, numbers of curious objects were seen, which, at first glance, might readily have passed for stages in the life cycle of a parasitic protozoan. It was therefore considered advisable to devote some attention to the phenomena displayed by normal blood, in order to clear up such possible sources of confusion. This work was done with dark-field illumination, and while the observations are perhaps in no case strictly original, they have been deemed worthy of presentation, if for no other reason than that they demonstrate the very high value of this method in microscopical research.

Objects seen but dimly or not at all with ordinary light are by this means brought out with perfect clearness. This is pointed out in a recent paper by Alfred C. Coles (1909). That author examined a given region of a preparation containing *Treponema pallidum* alternately with the dark-field illumination and ordinary light. This was done by changing the condensers without moving the slide. Over the dark field the organisms were plainly seen, but by transmitted light, in spite of the most careful focusing and adjusting of illumination, they remained wholly invisible.

My experience in the examination of fresh blood is quite similar, and warrants the conclusion that the use of the dark field is a *sine qua non* in such studies. Thus, we find reference in hematological literature to the presence of flagellated cells in fresh blood. Such, however, appear in most cases to have been looked upon as unusual occurrences and very difficult to demonstrate. This is no doubt the case when blood is studied by transmitted light, but when dark-field illumination is used flagellated cells are frequently and easily seen in fresh blood, as will presently be shown.

The apparatus used in these studies consisted of a substage condenser, arc lamp, and rheostat, the latter serving to cut down

the laboratory current to 4 amperes. In the use of this apparatus the top part of the condenser, the slide, and the cover-glass must all be scrupulously clean, and the film of oil between the condenser and the slide must be free of air globules. The mount, for the sake of the observer's eyes, should also be free from air globules, since they shine with the brilliancy of the sun when the light strikes them. It is stated in the literature published by the manufacturers of the dark-field illuminating apparatus that the slides used should not exceed 1 millimeter in thickness, and this seems to be a rather important point. Frequently the condenser has to be raised or lowered a little before the conditions become what they should, the criterion being the shade of the background, which should be black.

The work was done with a $\frac{1}{4}$ -inch achromatic immersion lens, stopped down with a hard rubber funnel. For the most part a No. 12 compensating eyepiece was used, giving a theoretical magnification of 1,440 diameters. Equally good pictures, however, were to be had with a No. 18 eyepiece, giving 2,160 diameters. In fact, the impression is that even much higher powers could be successfully used, since the details shown by the highest eyepieces were quite as clear as those seen with the lowest.

The blood studied was that of the cow, sheep, rabbit, guinea pig, white rat, and man. That of the cow and the sheep was drawn from the jugular vein, defibrinated, and preserved in sterile culture tubes. The media used were common beef bouillon, citrated salt solution, or salt solution alone. In a few cases no culture media were used at all.

In spite of the somewhat varied technique, the phenomena presented were throughout much the same. Citrated salt solution, however, made by mixing equal volumes of a one-half per cent solution of each of the constituents, appears to have a destructive influence on the blood cells when permitted to act upon them for any length of time. In bouillon, on the other hand, they maintained their integrity for many days.

It may also be noted that the dark-field illumination has an injurious action on living cells. This is doubtless due in part to the fact that that portion of the slide directly above the condenser is heated. The actinic rays of the electric light may also play a part. Parenthetically, it may be observed that trypanosomes perish very quickly when placed in such an environment.

PHENOMENA OBSERVED.

The phenomena observed are best treated under the following captions: (1) Blood dust; (2) beaded threads; (3) flagellated erythrocytes and free flagella; (4) bodies showing pseudopodia; (5) erythrocytes; (6) leucocytes; (7) blood plates.

BLOOD DUST.

Blood dust, so called, consists of rounded, elongated, dumb-bell or irregularly shaped particles varying in dimensions from elements 1 or 2 μ in diameter down to those just within the limits of the highest powers of the microscope. It has been described a number of times, and a résumé of the literature is given by Nicholls (1906). To this author also belongs the merit of giving us an excellent account of this element of the blood. His conclusions are that blood dust is not an element *sui generis*, but is of diverse origin. He gives as possible sources of origin the granules of dead or dying leucocytes, fragments of both erythrocytes and leucocytes, and finally precipitates from the plasma.

My own observations show that the dust is a constant constituent of the blood of all the animals studied. A drop of blood, either pure or in any of the several media used, shows a greater or less number of small dancing particles. These, as stated by Nicholls, are in some cases as large as 2 μ in dimensions, but they run down to particles so small that even under 2,160 diameters, and intensely lighted, they can be seen but as points of light in a ceaseless dance in the medium. There does not seem to be any means whereby the precise dimensions of such particles can be determined, but they are certainly far below the conventional and incorrect one-half micron which is often said to be the limit of microscopical resolution, for blood dust can be seen, against the dark field, with the 16 mm. objective and No. 2 compensating eyepiece, which gives a magnification of only 31 diameters. Under such powers it is easy to see that the field is full of dancing granules, although nothing can be made out as to their shape. Presumably, with this low magnification, only the larger granules are visible; that is, those approaching a size of 1 to 2 μ .

Under a magnification of 2,160, or some seventy-fold greater, particles of blood dust can be seen which are so small that they appear as mere specks of light. I am not certain that it is justifiable to regard these as only one-seventieth as large as those revealed by a magnification of 31, but they are certainly extremely minute, and probably but a small fraction of a micromillimeter in dimension.

As already stated, the larger particles of blood dust are round, oval, or rod or dumb-bell shaped. The smaller have always the appearance of solid bodies; the larger are either solid or vesicular.

While the dust was always present, it varied enormously in quantity in different animals and in the same animal at different times and under different conditions. Nicholls believes it to be more abundant after a meal. It was also observed that, in general, the longer a drop of blood was kept on a slide the greater became the quantity of dust. This goes to show that it increases in quantity as

the blood degenerates, which necessarily takes place if it be kept on a slide as a wet mount. On the other hand, a bouillon culture of cow's blood, kept for forty-seven days at room temperature, showed the erythrocytes much as they had looked on the first day, and approximately the same quantity of dust.

Since, as stated above, the object in making these researches had to do with the clearing up of possible sources of confusion with regard to the presence of protozoa, the question of the origin of the various elements seen was only of secondary interest and was not pushed to any conclusive end. Nevertheless certain observations were made which indicated at least one of the sources of blood dust. It was frequently seen that the particles tended to collect around the erythrocytes. In one case the erythrocytes—the blood being that of the guinea pig—were seen to be studded around their peripheries with a mantle of small granules, evidently of the same nature as those which were simultaneously dancing in the plasma. But in the guinea pig, cow, and man it was at times possible to make sure that there were free, motile granules *within* the red cells. The proof that they were within rested upon two points. In the first place, by shifting the focus from the upper to the lower surface of a given cell there was consecutively a less, a greater, and a less number of granules brought into view. That is, when the focal plane was on an optical section of the erythrocyte, the greatest number of granules was seen, which seems to shut out the possibility that they were on the surface. Secondly, when attention was concentrated upon a given red cell, it could be seen that the granules contained within its border showed a far less lively dancing movement than those which were free in the plasma. The only explanation seems to be that their movements were hampered by the bounding wall of the red cell. It therefore seems permissible to conclude that a certain portion of blood dust consists of broken-up erythrocytes or fragments derived from them.

After an experience of a couple of months with the dark-field illumination I became greatly impressed with its capabilities, and preparations were made of blood serum and bouillon which had been passed through a Chamberland B filter. Such filtrates showed the presence of great numbers of dancing particles, of the apparent size of the smaller particles of blood dust. It thus became evident that organic liquids, commonly looked upon as mere solutions and absolutely sterile to all bacteriological tests, contained corpuscular elements large enough to be resolved by the dark-field illumination. Nicholls also observes that serum taken from serous cavities contains the dust. We therefore see that a certain portion of the blood dust is not "blood" dust at all, in the sense of its being peculiar to blood,

but it is rather to be regarded in the nature of a precipitate and peculiar to all organic fluids. This fact has a certain bearing on the problem of the so-called "ultramicroscopic" germs of certain diseases; that is, the discovery of corpuscular elements in virus would be no warrant for concluding them to be the cause of the disease in question. It would be necessary to differentiate them from the so-called "dust," an undertaking which strikes one as rather discouraging.

BEADED THREADS.

These consist of strings of beads, or of threads showing septations, the appearance being somewhat that of a string of streptococci. While they may be seen by transmitted daylight, it is only in preparations studied over the dark field that their abundance becomes evident. They occur either attached (fig. 1, *a*) to erythrocytes or free in the liquid.

In the former case there may be one or many attached to the same cell, and they vary a good deal in appearance and dimensions. At times they are extremely delicate, with the septations scarcely to be made out, and here they closely resemble the "flagella" to be described below. This condition by gradual stages leads up to one wherein we find attached to a red cell a very evident string of beads. In nearly all cases the terminal bead is somewhat larger than the others. In length these attached strings may be very short, consisting of only two or three beads, or they may be two or three times longer than the diameter of an erythrocyte. The width of such strings is not easily measured, but it probably never much exceeds 1μ , and may be much less.

In all cases these attached strings are motile. The movement may be spoken of as flagellar, but it differs from a typical flagellar movement in that it is more regular and its rapidity more uniform. It more nearly resembles that of the tentacles of a sea anemone or of the various members of a colony of fresh-water polyzoa. The cause is doubtless the slight trembling of the erythrocytes—always to be noted in fresh blood mounts—combined with whatever influence it may be that produces Brownian movement. It is sometimes more, sometimes less, lively, but never either at all slow or especially quick.

In addition to the beaded threads attached to the red cells, abundant specimens are to be seen free in the liquid. The very exact resemblance between the free and the attached forms points to a common origin. The former, however, reach a much greater length, and in nearly all cases the two terminal beads are larger than the others. The length of one of these threads in human blood was 25μ , and there were others seen which considerably exceeded this measurement.

In their movements the free specimens are like those attached, in so far as the movements of a free body may be correlated with those of one which is fixed. There is a constant wavering and trembling of the whole thread and in addition a bending and looping movement. Fairly intricate figures are often formed by those which are being carried about by currents in the liquid.

There are a few observations which point to the mode of origin of these beaded threads. Frequently in the blood of most of the animals studied there could be seen groups of three or more vesicular, rounded bodies, varying in diameter from nearly that of a red cell downward. They had all the appearance of undoubted red cells, and my interpretation is that they were red cells in process of fragmentation. As a rule such groups showed one vesicle much larger than the others. There were also seen red cells to which were attached longer or shorter chains of little vesicular bodies, round or oval in contour. In general, the vesicles composing such chains were much of the same size in the case of the same cell, but showed a considerable variation for different cells. But this is not always the case, as shown by figure 1, *b* and *c*, drawn from appearances seen in the blood of the cow.

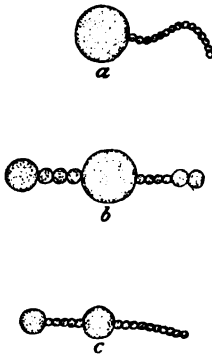


FIG. 1.—Beaded threads in blood. *a*, erythrocyte with attached beaded thread; *b*, erythrocyte to which are attached two strings of vesicles; *c*, element consisting of vesicles and beaded threads. Magnification about 1,100.

In the former of these we have an erythrocyte to which is attached two strings composed of the vesicles just described. Of these, some are relatively large, others are nearly as small as the beads. In figure 1, *c*, is shown a system composed of two large vesicles and two strings of beads. The latter, which always appeared to be solid, might be interpreted as derived from the former by a process of budding or by a mere contraction in size. It is therefore not unreasonable

to suppose that the beads are derived from the vesicles, while the latter are budded-off portions of the stroma of the red cell.

The threads were first discovered in a bouillon culture of cow's blood six days old. They were also found in a like culture forty-seven days old. They appeared, however, in preparations of blood of all the animals used. But whereas blood dust is constantly present in blood as soon as it is drawn, it was not possible to get any evidence that this is so for the threads. The quickest observed to appear was in human blood, when one was seen seven minutes after the blood was drawn. It was also noticeable that in blood drawn on slides, either clear or in citrate, free threads were seen much sooner than those attached, and that in general it was easier to find them the longer the mount was kept. But the number of free threads and of corpuscles

to which they were attached is very small in proportion to the whole number of red cells present, and hence the finding of free threads sooner than those attached might be due merely to accident.

FLAGELLA.

In bovine blood known to harbor *Piroplasma bigeminum*, kept in bouillon in the incubator for six days, erythrocytes were found to which were attached very delicate flagella, in constant, rather lively, lashing movement. This suggested the possibility that these represented the microgametes of *P. bigeminum*, and that in *Piroplasma* reproductive processes occur similar to those which take place in *Halteridium*. Unfortunately for this interesting hypothesis, exactly the same sort of flagellated cells were observed in blood from a northern cow which had, so far as was known, never been exposed to infection with *Piroplasma*, and later in that of all the other animals used.

But they were best demonstrated in sheep's blood, drawn direct from the jugular vein into sterile test tubes containing citrated sodium chlorid solution. In mounts from this material the appearances were at times astonishing, the entire field being filled with delicate, motile threads, either free or attached to the red cells. In the latter case the flagella usually arose from little V-shaped projections, and a given cell might support several. Their thickness was infinitesimal, and the field had to be very black and the light intense before they could be easily seen. In length they exceeded by six to eight times the diameter of the blood corpuscles.

In blood from the other animals flagellated red cells and less frequently free flagella could usually be found, but they were never present in anything like such abundance. The assumption is natural that the free flagella were merely those which had broken loose from their attachment to the cells, although this was never actually observed to take place.

I was not able to satisfy myself that the flagella were the end stage of the beaded threads. There is not much doubt, however, that they represent some sort of destructive process. As stated, they were most abundant in blood preserved in citrated sodium chlorid solution, which, for other reasons, was shown to have a destructive influence. They have been seen before, and are figured by Lignières (1900a).

BODIES SHOWING PSEUDOPODIA.

Koch (1906) and Kleine (1906) describe and figure bodies with long pseudopodia which they interpret to be stages in the evolution of *Piroplasma*. Koch illustrates these in his Plate I, figures 7-16, the material having been obtained from engorged ticks taken from cattle suffering with Texas fever. They were seen only during the first day

after removal from the cow. A slow ameboid creeping movement is described, with an alternate increase and decrease in the length of the pseudopodia. On Plate 3, figures 51-63, Koch figures similar bodies as developmental stages of the parasite of East Coast fever, *P. parvum*.

Kleine took defibrinated blood from dogs infected with *Piroplasma canis*, and cultured it at 27° C. after dilution with one-half per cent sodium chlorid solution. Preparations taken from such cultures eighteen to forty-eight hours after making showed bodies in all respects like those described and figured by Koch. (See Kleine, Pl. 4, figs. 6-13; Pl. 5, figs. 14-21.) In fresh mounts they were seen to change shape and the pseudopodia to increase and decrease in length and numbers. Christophers (1907), in his elaborate article on the cycle of *P. canis* in the tick, was not able to find bodies in any way resembling these.

In cultures, in either bouillon or citrated salt solution, of blood from either northern or southern cattle there were found bodies somewhat smaller than erythrocytes, round or oval, which bore from one to several long stiff processes. These bodies were not observed to move spontaneously nor to undergo any conspicuous changes of shape. A false impression of life was given them, however, by the fact that the processes bent and wavered and underwent changes in length. The bodies themselves were either homogeneous or granular.

They were first found in blood from a southern cow known to harbor *Piroplasma bigeminum*, and were identified as being the same as the bodies described by Koch and Kleine. Their subsequent discovery in blood from northern cattle showed, however, that they could have nothing to do with the Texas fever parasite, nor were they found in preparations made from engorged ticks.

It may therefore be concluded that they are only greatly altered blood cells; erythrocytes more probably than leucocytes, for the "pseudopodia," although very long and often very delicate, have something of the appearance of very much elongated thorns and do not resemble those often seen emerging from undoubted leucocytes. It may finally be pointed out that the German investigators fail to state whether their results were checked with material known not to contain *Piroplasma*.

ERYTHROCYTES.

As already stated, in bouillon cultures of cow's blood the red cells maintain their integrity for at least forty-seven days, but a drop of blood, either pure or mixed with citrate solution and studied with the dark-field illumination, shows quite rapid alterations.

Erythrocytes when first seen under such conditions are either perfectly circular or more or less crenated. In either case, however, the periphery appears as an intensely bright band with a more or less

yellowish color. In the circulation red cells are no doubt always circular, while crenation is the expression of some disturbance in the osmotic pressure between the cell and the medium in which it floats. But when pure blood either from man or from the guinea pig was kept over the dark field the originally bright red cells were observed to undergo the following changes: In those which were crenated when first brought under observation the irregular contours gradually became smoothed out, the cell finally showing as a bright circle, or rather as a bright thick ring. Then more or less suddenly they became pale, turning each to a transparent shell bounded by a faint bluish ring. These shells would sometimes fade out of sight altogether, but they mostly remained as such, and frequently there appeared within them dancing granules. Changes of this sort would take place within a few minutes, but were not always displayed by all of the cells under observation. In a drop of human blood, kept for twenty-four hours, the red cells were all reduced to these pale shells, most of which showed dancing granules within.

Curious phenomena were observed when blood was treated with one-half per cent solution of chlorate of potash. Here, as already noted, the originally bright crenated red cells gradually became perfectly circular, with a pale periphery. But in certain of these, the first instance being noted fifty minutes after the preparation was placed under the microscope, there appeared within the center a shimmer of light, and as this was watched it gradually resolved itself into a mass of excessively minute granules in a ceaseless lively dance. By slow degrees the granules became larger and larger, their movements meanwhile becoming less energetic, and finally the originally empty shell was filled with a solid mass of very bright motionless granules. This stage was reached in some cases within an hour or two, some cells undergoing the change much more rapidly than others.

In one or two cases in human blood a process somewhat the reverse of this was observed. Red cells were seen in which were a few quite large granules, or more probably little vesicles, showing a slight motility. These bodies drew together in the center of the cell and gradually fused to constitute a very irregular body, which, in its turn, became round, oval, or kidney-shaped, with smooth outlines. The outlines of these bodies were observed to undergo slight changes, and the bodies themselves to move slightly within the red cells. The appearance presented was of a cell within a cell, and although such a genesis as above described was seen in but a few cases, such elements within erythrocytes were often noted. The *modus operandi* suggested that we had to do with globules of either a liquid or a gas.

But several times while such cells were being watched the central body or vesicle was seen to grow smaller and then very quickly to

transform itself into a little nebulous cloud and as quickly to fade into nothingness. I am not prepared to explain the meaning of this phenomenon.

LEUCOCYTES.

As is well known, leucocytes are much less hardy than erythrocytes, although they live in culture tubes for a number of days. Under the severe condition above the dark-field condenser, however, they quickly perish. In an observation made on rabbit's blood the small lymphocytes were seen rapidly to disintegrate, becoming pale shells with a few dancing granules within. The polymorphonuclear leucocytes of man run down rapidly to masses of granular débris, while the nucleus in each case breaks up into a number of round bodies with a granular content. Nevertheless they can be kept intact long enough to observe very interesting phenomena. Living leucocytes throw out pseudopodia, processes intermediate between pseudopodia and flagella, and finally actual flagella, although this last was rather rare.

The pseudopodia were of the heliozoan type, enormously long, delicate strands, which underwent rapid changes in length, swung to and fro like a pendulum, and at times lashed. The heat of the dark-field illumination evidently stimulates leucocytes to great activity, and they were observed to travel rapidly about the field, either threading or forcing their way through the masses of erythrocytes. Such cells as they progressed became somewhat elongated, with the rear end pulled out into a sort of tail, from the end of which protruded a bundle of filamentous pseudopodia. In its advance the cell would frequently make rather sharp turns to the right or left, whereupon the entire congeries of pseudopodia would be quickly swung through an extensive arc. Further, individual pseudopodia could be seen to swing through wide arcs, often to bend into curves, to crinkle and waver, and to lash like flagella. Also as the pseudopodia were pulled along behind the leucocyte they would become attached to erythrocytes and greatly stretched. Finally, the strain becoming too great, they would break loose, snap back toward the leucocyte, curling up like elastic threads, the red cells to which they had been attached simultaneously showing a recoil.

BLOOD PLATES.

Blood plates are always to be seen in freshly drawn blood, and for a time in cultures. In the former they may be as numerous as the red cells. They are not favorable objects, however, for the dark-field illumination, and are best studied by transmitted light.

In form they are often tailed, and it is possible that an observer wholly unfamiliar with the appearance of flagellates might confuse

them with such. But they were not seen to display spontaneous movements, although a false appearance of this was occasionally given as they were carried about by currents in the mount. Stained preparations showed them both within and emerging from erythrocytes.

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II. THE PRIORITY OF CRYPTOBIA LEIDY, 1846, OVER TRYPANOPLASMA LAVERAN AND MESNIL, 1901.

In the Proceedings of the Academy of Natural Sciences of Philadelphia for September, 1846, Dr. Joseph Leidy (1846 a) describes a new genus and species of "Entozoa" as follows:

Cryptobia. Animal minute; form exceedingly proteoid; internal organization cellular or granular.

C. helicis. Colorless; form, ordinarily elongate, ellipsolid, fusiform, or ovate; caudated, caudæ opposite, one longer than the other. Internal granular structure consisting of two large cells and numerous minute granules. Total length from the one hundred and twenty-sixth to the one hundredth of a line. Habitat, the vesicæ copulatrice or spermatheca of *Helix albolabris* [*Polygyra albolabris* (Say)], *Helix tridentata* [*Polygyra tridentata* (Say)] and *Helix alternata* [*Pyramidula alternata* (Say)].

The name of this genus is derived from κρυπτος, hidden, and βιωω, to live. This singular entozoon in its general appearance and organization appears to be intermediate between *Cercaria semini* and *Filaria*. Its varied form and movements are curious to observe; at one moment globular, then oval, ovate, fusiform, sigmoid, crescentic, etc., it appears as if it would outvie the kaleidoscope in its changes. The motions are vibratile, rotary, with a lateral progression, or whirling in circles like the insect *Gyrinus*.

This article is illustrated by a large number of small text figures, which show that Leidy was dealing with an organism fundamentally fusiform and which possessed a flagellum at either end. One of his figures shows a bundle of four individuals in which the flagella from one end are joined by their tips.

In the same journal for August, 1847 (Leidy, 1847 d), we find it stated that "Doctor Leidy requested permission, which was given, to change the name of a new genus of Entozoa, described by him in vol. 3, No. 5, of the Proceedings, from that of *Cryptobia* to *Cryptoicus*, the former name having been preoccupied."

In the same year (Leidy, 1847 f) a description of the animal is given in Latin, but this is nothing more than a translation of the account quoted above. We learn, however, that in addition to the three hosts already named, it was also found in *Helix elevata* [*Poly-*

gyra elevata (Say)], *Helix thyroidus* [*Polygyra thyroidus* (Say)], and *Bulimus decollatus* [*Rumina decollata* (Linné)], and that—

This singular entozoon is a polygastric animalcule * * *. Sometimes it is collected in bunches adhering by the end of one of the cauda to each other, and frequently it may be observed to contract upon either of the long cellules, causing them to project beyond the outline of the animal * * *. In the collapsed state of the genitalia of *Helices* I could not detect the *Cryptocicus*.

Diesing (1850 a), vol. 1, p. 45, calls the parasite *Bodo* (*Cercomonas*) *helicis*, appending his own name. After giving the names of the three snails first mentioned by Leidy as hosts, and the locality as Philadelphia, he adds "*Helix nemoralis*, Vindobonæ, Augusto (Leidy)." The inference here is that Leidy found the parasite in *H. nemoralis*, at Vindobonæ, in August, but Leidy himself is silent.

Later, Leidy (1851 d and 1856 b) accepts Diesing's action in placing the animal in the genus *Bodo*.

Keferstein and Ehlers (1860) describe an "infusorian" in the bursa copulatrix of *Helix pomatia* Linné. It is present in great numbers. The organism is spindle shaped, bearing in front a lashing flagellum, while behind it is pulled out into a process about 8 μ long. The length is given as 20 μ , the width as 4 μ . The organisms are not round but flattened, and most of them show a longitudinal ridge. The content is finely granular, and after treatment with acetic acid shows an evident nucleus. The movements are mostly trembling and wavering, with a rotation on the longitudinal axis. Progression is in nearly a straight line. Occasionally some individuals were seen to creep in the manner of *Euglena*.

For close on to half a century *Cryptobia* remained undisturbed by science, but in the present year Friedrich (1909) picked it up in the "receptaculum seminis" of *Helix pomatia* L., and gives a detailed account of it. Following this, the animal possesses two flagella, of which the posterior makes up the border of an undulating membrane. Both arise from the kinetonucleus, which is of approximately the same size as the trophonucleus. Friedrich, after going into the history of the animal, decides that it is the same as that found by Leidy, and calls it *Trypanoplasma helicis* Leidy.

Clearly, however, if the later authors were dealing with the same animal as Leidy, the rules of priority compel the use of the name which he gave, and the generic name *Cryptobia* supplants that of *Trypanoplasma*. The only possible question which might arise would be whether or not *Cryptobia helicis* and *Trypanoplasma borreli* (the type species of *Trypanoplasma*) are congeneric.

In order to determine this point, living specimens of several species of snails were obtained from the neighborhood of Washington, D. C., and in *Polygyra albolabris* the flagellate was found. It is shown in

figure 2. From this it will be seen that the animal possesses two flagella, which arise from two minute granules in close proximity to the kinetonucleus. One of these flagella forms the edge of an undulating membrane. The kinetonucleus is of much the same size as the trophonucleus, but it may vary a good deal in shape, being at times crescentic. Friedrich considers the structure to be a good deal the same in both kinetonucleus and trophonucleus, but says that in the former the stain is generally so intense that the details are obscured. Nuclear structure, however, depends so much upon the conditions, and it is yet so obscure in the case of the Trypanosomidæ, that it is not advisable to use it as a diagnostic character of value.

Laveran and Mesnil (1907) define the genus *Trypanoplasma* as follows:

Trypanoplasma, Laveran and Mesnil, 1901 (amended 1904): Flagellates with elongated body, presenting laterally an undulating membrane, whose thickened edge is prolonged posteriorly as a flagellum, and turns back anteriorly to reach a mass (centrosome) which is as large as, and has up to a certain point the same structure as, the nucleus. An anterior free flagellum has its origin in the same mass. Multiplication is probably by binary equal longitudinal division.

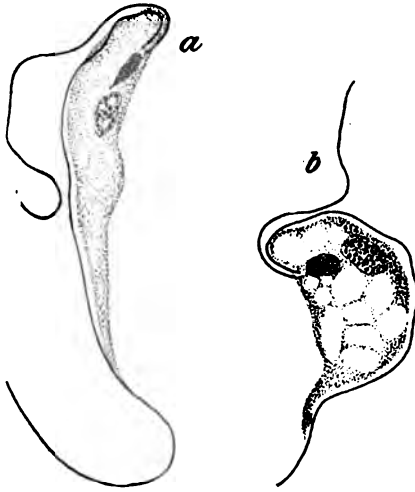


FIG. 2.—*Cryptobia helix* Leidy. *a*, typical form, $\times 2,467$; *b*, short, broad form, possibly somewhat degenerate, $\times 2,467$. (Figures made from camera outlines.)

This definition evidently covers the parasite of the snail. Further, a comparison of figure 2, *a* and *b*, with the drawings of *T. borreli* as given by Woodcock (1909, p. 215) shows plainly enough that that animal and *C. helix* are congeneric. It may further be added that Friedrich reports longitudinal division, a conclusion that is confirmed by my own material. Hence the name *Trypanoplasma* must give way to that of *Cryptobia*.

This name, originally used by Leidy, was afterwards abandoned by him on the score of its preoccupation by *Cryptobium*. But while *Cryptobium*, and *Cryptobias* as well, were used in 1830 and 1834, respectively, for beetles, this is no warrant for abandoning *Cryptobia*. For although these three names are very similar, they are not identical, and hence can not be regarded as homonyms.

The synonymy of the genus *Cryptobia*, and of the several species belonging in this genus, is given in the following table:

Genus **CRYPTOBIA** Leidy, 1846.

1846. *Cryptobia* Leidy, 1846a, p. 100 (type *C. heliciis* Leidy 1846). (Not *Cryptobium* Mann, 1830, Coleoptera; not *Cryptobias* Serv., 1834, Coleoptera; not *Cryptobia* Desh., 1863, Mollusca.)
1847. *Cryptoicus* Leidy, 1847d, p. 239 (*Cryptobia* renamed).
1891. *Trypanomonas*^a Labbé, 1891c, p. 231 (type *Trypanomonas danilevskyi* Labbé, 1891).
1901. *Trypanoplasma* Laveran and Mesnil, 1901i, p. 672-673 (type *T. borreli* L. & M., 1901).

Cryptobia heliciis Leidy, 1846.

1846. *Cryptobia heliciis* Leidy, 1846a, p. 100, figs.—Leidy, 1847f, p. 67, figs.
1847. *Cryptoicus heliciis* Leidy, 1847f, p. 67, figs.
1850. *Bodo heliciis* Diesing, 1850a, p. 45.—Leidy, 1851d, p. 284.—Leidy, 1856b, p. 42.—Kent, 1880c, v. 1, p. 256, pl. 14, figs. 12, 13.—Doflein, 1901a, p. 73.
1909. *Trypanoplasma heliciis* Friedrich, 1909, pp. 363-395, figs. 1-48.

Cryptobia abramadis (Brumpt, 1906).

1906. *Trypanoplasma abramadis* Brumpt, 1906, p. 164.

Cryptobia barbi (Brumpt, 1906).

1906. *Trypanoplasma barbi* Brumpt, 1906, p. 164.

Cryptobia borreli (Laveran & Mesnil, 1901).

1901. *Trypanoplasma borreli* Laveran & Mesnil, 1901i, p. 672, fig. 4; p. 673.

Cryptobia cyprini (Plehn, 1903).

1903. *Trypanoplasma cyprini* Plehn, 1903a, pp. 175-181, pl. 2.

Cryptobia danilevskyi (Labbé, 1891).

1891. *Trypanomonas danilevskyi* Labbé, 1891c, pp. 229-231, figs.

Cryptobia guernei (Brumpt, 1905).

1905. *Trypanoplasma guernei* Brumpt, 1905, p. 322, fig. 34 (2); p. 323; p. 330, fig. 38 (1).

Cryptobia intestinalis (Léger, 1905).

1905. *Trypanoplasma*^a*intestinalis* Léger, 1905e, p. 512.

Cryptobia truttæ (Brumpt, 1906).

1906. *Trypanoplasma truttæ* Brumpt, 1906, p. 164.

Cryptobia varium (Léger, 1904).

1904. *Trypanoplasma varium* Léger, 1904s, p. 345.

Cryptobia ventriculi (Keysselitz, 1906).

1906. *Trypanoplasma ventriculi* Keysselitz, 1906, p. 37, figs. 46-48.

^a Labbé credits this name to Danilevsky, but the latter author (1889a, p. 66) used it merely to designate young stages of trypanosomes, and hence did not establish it as a valid generic name.

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III. TRYPANOSOMA AMERICANUM N. SP., A TRYPANOSOME WHICH APPEARS IN CULTURES MADE FROM THE BLOOD OF AMERICAN CATTLE. (PRELIMINARY NOTICE.)

HISTORICAL.

In a postscript to his celebrated article on the blood parasites of the Little Owl, Schaudinn (1904) details some information received from Kossel and Weber in a personal communication. The last-named investigators, in Finland, in 1900, had made a smear of blood, taken from a cow dying of hemoglobinuria. In this smear, in addition to examples of a *Piroplasma*, which they identified as *Piroplasma bigeminum*, there was found a trypanosome, considerably smaller than *T. evansi*. Schaudinn examined this preparation, and after comparing the morphology of the trypanosome with that of *Piroplasma bigeminum* states that it probably constitutes a stage in the life history of *P. bigeminum* similar to that taken by the trypanosome of *Halteridium* in the life history of that blood parasite. His belief was based on the size, character of the cytoplasm, and nuclear relations of the two phases found in Kossel and Weber's preparations.

The year before, Kossel and Weber had made smears of the intestinal contents of ticks taken from sick cattle. On account of the above discovery these smears were restained and studied, and in them were found trypanosome-like stages similar to those found in the blood of the cow. Hence, with Kossel and Weber, Schaudinn accepts as a working hypothesis that the evolution of *P. bigeminum* and *P. canis* takes place in a manner similar to that of *Halteridium*.

Bowhill and Le Doux (1904), in a paper on malignant jaundice of the dog, refer to endoglobular parasites which show long, flagella-like processes. It is also stated that one of them (Bowhill) has observed flagellated forms of a *Piroplasma*, which they call *P. bovis*. They write: "The parasite consists of an enlarged elongated portion running into a delicate 'flagellum' upon which, on close examination, may be seen two minute bulbous protuberances."

Bowhill (1905), studying equine piroplasmosis, finds, free in the blood stream, parasites which "consist of a delicate, pear-shaped head, possessing a clearly defined red-stained karyosome, and a long flagellum ending in a bulbous protuberance." He adds that these

flagellated forms may be within a red blood cell, or half in and half out. The flagellum may be $3.5\ \mu$ in length or even more.

A glance at the figures given by these authors shows, however, that they are not dealing with flagella at all, but with processes resembling pseudopodia. This is pointed out by Minchin (1909), who writes (p. 89):

I suggest that the "flagellum" is probably to be interpreted as a pseudopodium, by means of which the parasite may quit a corpuscle, and by means of which it may, when free, attack and enter another corpuscle. It is not possible, however, to give a final judgment upon this point until the so-called flagellum has been seen and studied in the living condition.

Miyajima (1907) gives what seems to be excellent evidence in support of the doctrine that trypanosomes and Hæmosporidia are but phases in the life history of the same organisms. Cultures made in common bouillon of blood from Japanese cattle parasitized with a species of *Piroplasma* presently developed trypanosomes. The history of such cultures is as follows:

First day. No motile forms are seen.

Second day. A number of motile cells appear which occupy the upper layer of sedimented corpuscles and which show macroscopically as whitish dots.

Third day. A few motile forms are seen.

Fourth day. Motile forms, typical trypanosomes, appear in numbers, multiply rapidly, and reach their maximum in from ten to fourteen days.

In cultures at room temperature the trypanosomes remain motile for forty-five days. At from 10° to 20° C. they live for three months. Subcultures are also readily made.

These trypanosomes Miyajima regarded as derivatives from the *Piroplasma* present, for the following reasons: In the first place, the blood of 200 cattle was examined for trypanosomes, without result, and trypanosomes have never been seen in Japanese cattle. He then selected 21 native animals and examined their blood. In 9 of these *Piroplasma* was found; in 12 it was not. Cultures were then made of the blood of these 21 animals, and trypanosomes appeared in 7 cases, all from animals the blood of which had previously shown *Piroplasma*. The remaining 14 cultures were negative.

Further to prove his results, Miyajima tried the following experiment: Three calves were selected the blood of which failed to show parasites. These were injected with a culture containing motile trypanosomes in abundance, and later were kept free from ticks. One animal gave negative results, but the other two became infected, as was proved first culturally and later microscopically. Miyajima writes (p. 90):

In one instance, eight days after inoculation, the blood of the susceptible animal began to give a growth of the flagellated parasite in culture, whereas seventeen days later, by the aid of the microscope alone, the intracellular parasites were visible in the same animal.

It was found that the quantity of blood used to inoculate the tubes was a matter of indifference. One platinum loopful gave precisely the same results as 1 cubic centimeter.

Miyajima's conclusions, given on page 90, are here quoted:

1. A variety of hæmocytozoa known as *Piroplasma parvum* can readily be cultivated outside of the living body.
2. The parasites undergo the developmental change in blood bouillon and finally take the form of a typical *Trypanosoma*. This *Trypanosoma* can not be detected in the blood of living animals.
3. A simple mixture of blood and bouillon is the most suitable medium for the cultivation of protozoa such as *Piroplasma parvum* and *Trypanosoma lewisi*.

In an earlier communication Miyajima, in collaboration with Shibayama (Miyajima and Shibayama, 1906), brought out the following facts: From 13.6 to 76.1 per cent of Japanese cattle, according to locality, were found to be parasitized by a *Piroplasma*. It was not possible, by blood injections, to transfer this parasite from one animal to another. There was no evidence to show that this parasite is pathogenic to either native or foreign cattle. In the case of the former the only method whereby the presence of the parasite could be demonstrated was by microscopical examination. As to the latter, the authors examined a herd of 20 cattle imported some time previously from Wisconsin. Although all of these animals had remained in perfect health, 14 were found to be parasitized. On the other hand, two Japanese calves (age not given) were infested with ticks hatched from infectious eggs received from the Bureau of Animal Industry. The eggs were from ticks collected in Texas. The two calves, each infested with 200 ticks, responded by a mild attack of what was presumed to be Texas fever, but with regard to the blood examination the authors wrote: "Während die ganzen Beobachtungszeit zeigte der tägliche Parasitenbefund bei der Versuchstieren keine Abweichung von dem normalen Kalbes." From which the supposition is that *Piroplasma bigeminum* was not found, but that the Japanese *Piroplasma* remained present as in the control animals.

From the above it is evident that the Japanese *Piroplasma* found by these authors is peculiar in that it is not pathogenic. In this respect it differs from all of the other known species of this genus. Whether this is any warrant for believing that it is really not a *Piroplasma* at all is of course very questionable, but it may be pointed out that it does not follow that all of the pear-shaped parasites of the red blood cells of mammals are genetically connected. How this may be can not be determined until the life history of each species is known. I call attention to this fact here, since Miyajima's work is quoted as showing that *Piroplasma* has a trypanosome stage in its life history, a conclusion scarcely warranted.

In addition to the above papers, which are concerned with the attempt to link together trypanosomes and Hæmosporidia, there are a considerable number of observations which prove the presence of trypanosomes in cattle where there is no reason to believe them pathogenic.

Frank (1909), in Germany, found living specimens in the spleen pulp of an ox, the animal having died of a disease taken to be either anthrax or blackleg. The bacilli of neither of these diseases being found, Frank was inclined to blame the animal's death on the trypanosome, but no proof was furnished.

Frosch (1909) describes this parasite, which he calls *Trypanosoma* Frank, his material being stained smears from the spleen. It shows a sharply pointed posterior end, often terminated by a flagellum; a round, peripherally placed kinetonucleus, between which and the trophonucleus there is a wide interval. Frosch agrees with Frank in considering the trypanosome to be pathogenic.

Frank and Frosch (1909) restate the proposition of the pathogenicity of this parasite, saying that this is the first case of a bovine dying in Europe of trypanosomiasis.

Knuth (1909), studying the same material, made measurements of the flagellates present. He gives the length as from 20 to 40 μ , which includes the flagellum; the breadth as 2 μ . According to this author, it more closely resembles *T. theileri* than any other species.

Mayer (1909) also discusses this same trypanosome, and after going over its morphology says that it has the characters of a certain group of trypanosomes, typified by *Trypanosoma theileri*, which are found exclusively in cattle, hitherto only in tropical or subtropical countries.

Mayer then quotes a number of authors who have found trypanosomes occurring sporadically in cattle. Such mostly show the characters of this group. These discoveries were made in Asia and Africa. In some cases the trypanosomes were in healthy cattle; in others in those suffering from various diseases other than trypanosomiasis. Among these may be mentioned piroplasmosis and rinderpest. In no instance was there any reason to look upon the trypanosomes as pathogenic.

Hence Mayer regards the animal discovered by Frank to be harmless. Finally, he calls it *Trypanosoma franki*, a valid name.

METHODS.

Miyajima's work was repeated in this laboratory with entirely satisfactory results. Bovine blood, cultured in common beef bouillon, develops trypanosomes in from two to four days, dependent upon temperature. They also appeared in cultures of cow's blood in mut-

ton bouillon, either acid or alkaline, and, furthermore, they developed in the case of every cow tested.

The methods were as follows: Blood was drawn from the jugular vein of the cow by means of a syringe, and transferred to flasks of 100 c. c. capacity. About 30 to 50 c. c. of blood was taken in each case. In each flask were placed 6 to 8 facetted beads of rough glass, such as those found in shops, strung together, for sale as very cheap necklaces. A few minutes shaking of the flask serves to collect the fibrin into a solid clot, embedded in which will be the beads. In these operations suitable precautions were taken to prevent contamination.

To take the blood from the flasks, pieces of glass tubing were used, one-fourth inch in diameter and 8 to 10 inches long. Over one end of each of these a piece of rubber tubing was pulled, to serve as a mouthpiece. The quantity of blood desired may then be drawn up into the glass tube by suction. The tubes with the rubber tubing attached were sterilized by boiling for five to ten minutes and then used at once. Owing to the poor conductivity of glass, they do not cool readily, and it was considered possible that they might heat the blood sufficiently to kill any animal life present. Accordingly, the precaution was usually taken of allowing the first filling of the glass tube to drop back into the flask and of using only the second.

As soon as the glass tube was filled with the blood to be used, the rubber tube was closed by pinching and the contents transferred at once to a culture tube. This operation was always performed by two persons, one to handle the blood, the other the culture tubes. In this way the latter were not exposed to the possibility of contamination for more than a second or two.

This procedure gave entirely satisfactory results. Bacterial contamination, when it occurs, appears to be the result either of getting the blood contaminated during the process of drawing it, or in the later handling of the tubes. Moreover, it is not quite so destructive to the growth of the trypanosomes as some authors maintain. While the contaminating of cultures is a reflection on the operator's technique, the trypanosomes seem able to endure a certain amount of plant life, and may even be found alive in very foul cultures. It was also at times noted that bacteria would start in a tube and later die out.

OBSERVATIONS.

As a rule the trypanosomes appeared on the third day. In one case, however, the "room temperature" being high, from 80° to 90° F., they were found in forty hours. After a few days the microscope is not necessary to demonstrate their presence, since they collect in little colonies on the surface of the column of blood cells.

These colonies show as small, white plates, and may be 3 or 4 millimeters in diameter. They are readily distinguishable from bacterial contamination, in that they are flat and sharply circumscribed, whereas the masses of bacteria are always more or less diffuse and tend to cloud the bouillon.

The ratio of the quantity of blood to bouillon was varied within wide limits, but, as Miyajima found, this makes no difference as far as the appearance of trypanosomes is concerned.

So far as the study has yet gone, the flagellates have appeared in the blood of all the cows tested, seven in number. Of these, the first used was a cow born at the Experiment Station of the Bureau of Animal Industry at Bethesda, Md., on November 15, 1900. This animal had twice been injected with blood from cattle known to be parasitized with *Piroplasma bigeminum*. It was hence assumed that her blood contained this parasite, and it was for this reason that she was chosen for the experiment.

Accordingly, the appearance of the trypanosomes was taken to be an exact confirmation of Miyajima's results, to the effect that *Piroplasma* has a flagellate stage in its life history. But whereas the Japanese biologist had to depend upon the tedious and uncertain method of examination to determine whether or not he was dealing with animals parasitized by *Piroplasma*, this point can in the United States be settled without difficulty and with almost the certainty of a mathematical demonstration. The cattle in the northern parts of the United States, outside of the tick-infested region, are free from *Piroplasma*. This can be proven in individual cases by placing infectious ticks on the animal—i. e., ticks which have hatched from eggs laid by ticks taken from southern cattle. A northern cow thus treated will sicken of Texas fever and usually die, whereas no effects will be noticed in the case of a cow in the blood of which *Piroplasma* was already present.

Therefore, four animals were obtained from north of the Federal quarantine line, and cultures made of their blood. The trypanosomes appeared with due promptness. Since the chances were all against these animals being parasitized by *Piroplasma* (and, further, since one of them, as noted below, after being infested with ticks contracted Texas fever with fatal results), it was seen that there was a serious discrepancy between Miyajima's results and mine. My Japanese colleague got trypanosomes from blood in which he demonstrated the presence of a *Piroplasma*, and failed to get them in blood in which *Piroplasma* could not be found, whereas my experience is that the trypanosomes appear without reference to the presence or absence of *Hæmosporidia*.

As an additional test, two cows from the herd at the Experiment Station of the Bureau of Animal Industry were tried, and the flagel-

lates developed in cultures from their blood. The animals of this herd are assumed to be, and in all probability are, free from *Piroplasma bigeminum*. Finally, one of the four cows purchased for the experiment was infested with infectious ticks, and in due course died of Texas fever—practically absolute proof that at least one animal from the blood of which trypanosomes were cultured did not originally harbor the hæmosporidian. It was not considered necessary to sacrifice the other animals used in the study.

The first point which suggests itself is whether the trypanosomes which appear in culture tubes arise from actual trypanosomes originally present in the blood or from some element of a wholly different facies. While this point can not be regarded as finally settled, the probabilities are in favor of the second hypothesis. The blood of American cattle, in both health and disease, has been studied for many years, and with a single exception (Bowhill, 1909) trypanosomes have never been reported. If they were normally present in any numbers, it seems unlikely that they should so far have escaped notice. Moreover, while many of the cultures were examined daily from the first, flagellates were never found on the first day, and but once on the second.

But the most direct evidence that they evolve in the tubes from some unrecognized form is the fact that at first we do not find typical trypanosomes, or even flagellates, but round or oval bodies. Miyajima figures bodies which he regards as the forerunners of the flagellates, but it is not certain that these are not white blood cells. Neither his figures, unfortunately made by photography, nor his description permit of a final judgment.

In my own preparations I have now and then come across an isolated spherical body, of much the size of a large leucocyte, which does not exactly resemble any of the various types of white cells. It may be that such belong to the cycle of the trypanosomes, but to settle this requires further investigation. At present I am only able to say that I have not been able to get hold of anything which can safely be put down as a forerunner of the trypanosomes until the body shown in figure 3, *a*, appears.

This condition may be seen in young cultures. Such masses consist of closely compacted cells which are probably round or oval, but which are sometimes polyhedral on account of mutual pressure. They stain blue in Wright's stain, and each shows a distinct kinetocore, but trophonuclei are not to be made out. Clusters of this sort vary greatly in size. They may consist of as few as three or four organisms or as many as several hundreds.

This leads up into the condition shown in figure 3, *b*. Here is a cluster of little flagellates, of which the cytoplasm is still sharply

blue and the kinetonuclei very distinct. Flagella may or may not be present. Apparently the organism evolves from a rounded cell into a lenticular flagellate, but in some cases the lenticular form

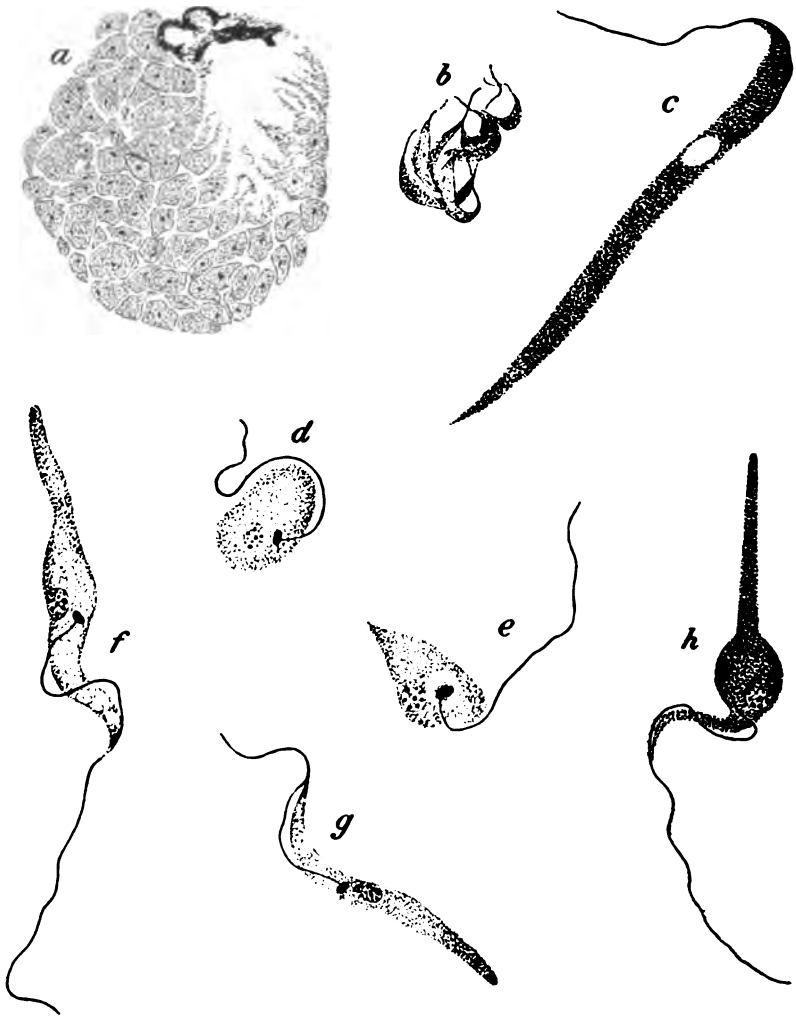


FIG. 3.—*Trypanosoma americanum* n. sp. *a*, cluster of young forms, 80-hour culture, X 800; *b*, cluster of small flagellated forms, 88-hour culture, X 800; *c*, crithidia stage, 88-hour culture, X 2,467; *d*, monadine form, 10-day culture, X 2,467; *e*, monadine form—the kinetonucleus lies in a vacuole stained pink by Wright's stain, 10-day culture, X 2,467; *f*, typical band-shaped trypanosome, with fully developed undulating membrane, 10-day culture, X 2,467; *g*, small band-shaped trypanosome, with fully developed undulating membrane, 10-day culture, X 2,467; *h*, club-shaped form, with fully developed undulating membrane, 10-day culture, X 2,467. (Figures made from camera outlines.)

may be assumed before the flagellum has differentiated. Small, isolated lenticular organisms are often seen which do not show flagella.

As a rule, however, these little bodies are typical flagellates, resembling *Crithidia* in morphology, and it is convenient to speak of this developmental phase as the crithidia stage. From this point on, in at least a considerable proportion of the cases, evolution consists apparently in a mere increase in size, till we reach the condition shown in figure 3, *c*. Here we have a long, band-shaped cell, with a sharply pointed posterior end. The specimen drawn was 28 μ long without the flagellum. The cytoplasm of these stages stains a clear blue, the kinetonucleus is very distinct, while the trophonucleus is represented by a pale, reddish vacuole, and is clearly very poor in nucleic acid. The flagellum is distinct, but an undulating membrane can not be distinguished in stained preparations.

In addition to the elongated crithidia forms, a monadine form also appears in young cultures, such as is depicted in figure 3, *d* and *e*. These monads become more abundant as the cultures grow older, but so far as my observations go at present they never form more than a small percentage of the trypanosomes present.

The typical trypanosomes are shown in figure 3, *f*, *g*, and *h*. In these the trophonucleus shows as a fair-sized vesicle containing coarse chromatin bodies. The kinetonucleus is usually elongated, forming a long ellipsoid, and stains an almost black garnet color. The two nuclei are always close together. The undulating membrane is well developed. As may be seen from the figures, the parasites appear under two forms, a band shaped and a club shaped. What significance this dimorphism may possess has not been worked out. It may be stated, however, that these two forms are readily to be distinguished from one another, although they to a certain extent intergrade. The band-shaped forms, or more typical trypanosomes, are more numerous than the others. At first glance the club-shaped phase might be looked upon as a degenerating condition, i. e., as a so-called involution form.^a But this can hardly be the case. The club-shaped animals appear in the cultures as soon as do the others, and neither their appearance in life nor their staining reactions in smears carries the least suggestion of degeneracy.

The specimens drawn in figure 3, *f*, *g*, and *h*, were of intermediate size. Larger individuals were seen, and the length over all may reach as much as 60 μ . In cases where degeneracy had apparently set in the posterior end was often greatly prolonged and now and then provided with a flagellum.

Division, in the cases noted, takes place by equal, longitudinal fission.

It will be seen from the above that the trypanosomes which appear in the culture tubes do not arise, so far as all appearances go, from

^a The use of the term "involution" as a synonym for "degenerate" is extremely unfortunate, but it probably has obtained too good a foothold in the literature to permit of the hope of its being dropped.

preexisting trypanosomes, but from larger or smaller clusters of round or oval cells, which in their turn are doubtless derived from some unrecognized form. Since the study is as yet by no means complete, it is necessary to speak with all reserve; but as long as the earlier cultures show only the clusters of rounded cells or flagellates of the crithidia type we appear to have a development from some condition which bears no resemblance to a flagellate, and which has not yet been recognized. It is only in the somewhat older cultures that typical trypanosomes appear, but these older cultures also show the adolescent stages, suggesting that all of the hypothetical germs present in a tube do not develop at the same rate.

In all the cultures the trypanosomes tend to occur in great clusters. Thus such a condition as is shown in figure 3, *a*, appears to give rise to a similarly constituted mass of trypanosomes in either the crithidia or trypanosome stage. The trypanosomes occurring free in the liquid are apparently individuals which have escaped from such colonies. These clusters, however, are not to be confounded with agglutination rosettes, which present a wholly different appearance. In the clusters nothing in the way of a definitive orientation can be made out, whereas in all the agglutinations seen the arrangement was radial.

Observations on the living trypanosomes were made with either ordinary light or the dark-field illumination. They perish so quickly with the latter, however, that its use is scarcely to be recommended. The trypanosomes are energetic enough, but seldom display a progressive movement. When they do, however, it is very rapid and may be either forward or backward. For the most part, however, the movements are confined to vigorous lashings of the flagellum, changes of the form of the body, and a sort of jumping hither and yon, which does not bring about any extensive change of shape. The club-shaped forms were often seen to display a very quick, sudden, jerky movement, accompanied by a shift in the direction of the longitudinal axis. It was like the movement frequently displayed by *Stylonychia* and related ciliates, only much quicker and more abrupt.

When the fresh mounts were of sufficient thickness as not to compress the large clusters, these, in mass, were seen to change outlines like slow amebas, and to shift their positions. They are covered with a mantle of very lively flagella and are so compact that they suggest a metazoan as much as they do a cluster of protozoans.

It may be concluded that the trypanosome here described and figured is a common parasite of healthy American cattle. Its morphological peculiarity is that the trophonucleus and kinetonucleus lie very close together. This peculiarity is shown by *T. transvaliense*,

taken to be a variety of *T. theileri*, and, as well as can be made out from his figures, by the trypanosome found by Miyajima. If this last fact be so, then Miyajima is in error in his conclusion that his flagellate is a phase of *Piroplasma*. At all events, the fact that trypanosomes appear in cultures of blood from healthy cattle is of considerable significance, and is decidedly against the belief that they are stages in the life history of a hæmosporidian.

Upon the completion of further investigations concerning this trypanosome, for which the name *Trypanosoma americanum* is suggested, a more extended account of its morphology and ontogeny will be given.

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BUREAU OF ANIMAL INDUSTRY.—BULLETIN 120.

A. D. MELVIN, CHIEF OF BUREAU.

THE INTRACELLULAR ENZYMES OF PENICILLIUM AND ASPERGILLUS,

With Special Reference to Those of
Penicillium Camemberti.

BY

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1910.

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LETTER OF TRANSMITTAL.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY,
Washington, D. C., October 9, 1909.

SIR: I have the honor to transmit herewith for publication a manuscript entitled "The Intracellular Enzymes of *Penicillium* and *Aspergillus*, with Special Reference to those of *Penicillium camemberti*," by Arthur W. Dox, chemist in cheese investigations in the Dairy Division of this Bureau. The experiments recorded in this paper have grown out of the work in connection with European varieties of soft cheese, which has been in progress for several years at Storrs, Conn., in cooperation between the Storrs Experiment Station and this Bureau. Much of the work referred to has been concerned with the manufacture of the Camembert type of cheese, and the characteristic mold of this cheese, *Penicillium camemberti*, was the organism used by Doctor Dox in the enzym experiments described herein.

I recommend the publication of the article in the bulletin series of this Bureau.

Respectfully,

A. D. MELVIN,
Chief of Bureau.

HON. JAMES WILSON,
Secretary of Agriculture.

CONTENTS.

	Page.
Introductory.....	7
Intracellular and extracellular enzymes.....	8
Molds in general.....	9
<i>Penicillium</i> and <i>Aspergillus</i>	9
Distribution and economic importance.....	10
Adaptability to artificial culture.....	11
Sources of carbon and nitrogen.....	11
Changes in the culture medium.....	13
Separation of racemic mixtures.....	14
Behavior toward antiseptics.....	15
Review of previously published work on the enzymes of <i>Penicillium</i> and <i>Aspergillus</i>	16
The various methods of preparing the enzymes.....	16
The particular enzymes found.....	17
Summary of the literature.....	35
Experimental work.....	35
Chemical differences in species of <i>Penicillium</i>	35
The organism selected, <i>Penicillium camemberti</i>	36
Choice of a culture medium.....	37
Methods of culture.....	38
Preparation of enzyme powder.....	38
Composition of enzyme powder.....	38
Experimental methods.....	39
Enzym studies.....	39
Summary.....	64
Acknowledgment.....	65
Bibliography.....	66

THE INTRACELLULAR ENZYMES OF *PENICILLIUM* AND *ASPERGILLUS*, WITH SPECIAL REFERENCE TO THOSE OF *PENICILLIUM CAMEMBERTI*.

INTRODUCTORY.

The importance of the lower plants in their relations to the various biochemical processes constantly going on about us is being recognized more and more as new data are accumulated. This field of research, which was first brought to the notice of scientists some fifty years ago, has proved one of the most interesting in the whole realm of biological chemistry. Although at the present time we are only on the threshold of any adequate conception of the various chemical processes which constitute life in its lowest forms, sufficient knowledge has already been gained to form a working hypothesis, which not only explains many of the well-known phenomena but also aids in the discovery of new truth. Perhaps the most striking example of this fact is to be found in the development of our knowledge of enzymes or unorganized ferments, especially the intracellular enzymes. Most of the processes upon which life and growth are so intimately dependent can now be regarded as more or less simple chemical reactions, in which an enzyme plays the part of a catalytic agent. Duclaux even goes so far as to suggest that the life of bacteria is probably nothing more nor less than the sum total of all the activities of the intracellular enzymes present.

Long after enzymes were known and studied in their relation to the digestion of food, chemical reactions of a similar nature which took place through the intervention of micro-organisms were explained upon entirely different grounds. Thus the distinction was made between organized ferments and unorganized ferments or enzymes, the former term being practically synonymous with living cells. Pasteur was the first to show that different types of fermentation were caused by specific organisms, and his epoch-making researches revolutionized the contemporary theories regarding fermentation. The former distinction between organized and unorganized ferments was finally broken down in quite recent years by Buchner. Claude Bernard had already suspected that the transformation of sugar into alcohol and carbon dioxide was due to the presence

of an enzyme acting within the yeast cells, but his attempts to prepare this enzyme had resulted in failure. Buchner, however, showed conclusively that alcoholic fermentation could be carried on in the absence of yeast cells by an enzyme prepared from the yeast.

Such enzymes which during the life of the organism act within the cell are now designated as endo- or intra-cellular enzymes. And it is by means of intracellular enzymes, prepared from the cells by suitable methods, that many of the reactions which were once thought to be absolutely dependent upon some mysterious vital activity are now carried out in the absence of living organisms.

INTRACELLULAR AND EXTRACELLULAR ENZYMES.

The distinction between intracellular and extracellular enzymes is in the main an arbitrary one. There is evidence to believe that the same enzyme may, in the earlier stages of growth of the organism, function as an intracellular enzyme, and later be liberated into the substratum as an extracellular enzyme. In the case of yeast, the amount of active sugar-fermenting enzyme or "zymase" set free in the medium during the normal course of organic growth is very small, the fermentation taking place mainly within the cells. This would account for the failure of the earlier observers to find any enzymatic activity in the medium after the cells had been removed. With molds the case is somewhat different. As the fruiting stage is reached a considerable amount of enzyme is liberated into the medium, and there is no particular reason for regarding this enzyme as different in origin and character from that remaining in the cell. For the sake of convenience, however, the distinction will be adhered to in this paper, as the work deals with the enzymes obtained directly from the mycelium.

The intracellular enzymes of certain groups of the lower plants have been investigated to a considerable extent, particularly the yeasts^a and bacteria.^b The researches on the enzymes of molds, however, have been of a more or less desultory nature, and the results, which are scattered over various chemical, biological, botanical, and mycological journals, have not as yet been collected. A great deal of this work has been carried out with organisms which have not been properly identified or where great confusion exists in the nomenclature. The results are in many cases somewhat uncertain, especially where insufficient data are given to make possible a repetition of the experiments. This point will be more fully discussed in the subsequent pages.

^a Meisenheimer, J. Ueber die chemischen Vorgänge bei den als Enzymreaktionen erkannten Gärungen. *Biochemisches Centralblatt*, 1907, Band 6, No 16/17, pp. 621-633. Leipzig, 1907.

^b Fuhrmann, F. *Vorlesungen über Bakterienenzyme*. Jena, 1907.

MOLDS IN GENERAL.

A study of fungi in general would, of course, be far beyond the scope of this paper. One great group, usually called the Hyphomycetes, contains many species with wide distribution as saprophytes upon all kinds of substrata. In this group are described some 5,000 species with diverse relationship but held together by one common character—lack of sexual reproduction. Certain of these cosmopolitan groups of species together with certain of the Phycomyces make up the aggregation to which the term “molds” is commonly applied. Phycomyces or conjugating fungi include the well-known Mucors, the Peronosporæ or rotting molds, the Cystopi or white rusts, and many other parasitic fungi, such as the Saprolegniaceæ or fish molds, and the Entomophthoraceæ or insect molds. Two other orders, the Uredinæ or rust fungi and the Ustilaginæ or smut fungi, are likewise of great economic importance. Very few of these molds have ever been studied from the standpoint of biological chemistry. The great bulk of the work has been taxonomic and pathological in character.

PENICILLIUM AND ASPERGILLUS.

The organisms selected for investigation by chemists not having an intimate knowledge of mycology would naturally be those of wide distribution and omnivorous habits. In some of the earliest work, when a mold culture was desired, the medium was simply left exposed to the air until spontaneous inoculation ensued from the spores floating about in the atmosphere. Cultures obtained in this way usually consisted of various species of two genera, *Penicillium* and *Aspergillus*, which are placed by Engler and Prantl in the family Aspergillaceæ. This family grouping is based upon certain species of each group for which sexual stages have been found, without necessarily implying that all described species of these two genera belong together. In fact, as these two genera are at present constituted, most of the described species belong rather to the Hyphomycetes than to the Aspergillaceæ as considered by Engler and Prantl.^a

The main morphological difference between *Aspergillus* and *Penicillium* is in the appearance of the fruit bodies. In *Aspergillus* the aerial filaments bearing the spores are generally formed of a single cell which swells out at the end into a sphere. Numerous cells radiate from this sphere, each bearing one or more chains of spores. In *Penicillium*, on the other hand, the aerial filaments send out branches which ramify again, forming conidiiferous cells, and these terminate in chains of spores. The general appearance is that of a

^a Engler, A., and Prantl, K. Die natürlichen Pflanzenfamilien. Leipzig, 1887.

broom, whence the name *Penicillium*. While various species of these two genera may be closely related morphologically, they often show wide chemical differences. In fact some of them may be distinguished more readily by their chemical properties than by the aid of a microscope.

DISTRIBUTION AND ECONOMIC IMPORTANCE.

Penicillium and *Aspergillus* are both very widely distributed in nature. Almost any nutritive substance which contains a sufficient amount of moisture and is not positively toxic will, if left exposed to the air for a short time, become infected with species of one or both of these genera. Everyone is familiar with the appearance of moldy bread, fruit, or cheese. And almost any solution containing carbon compounds which do not have antiseptic properties soon becomes covered with a growth of these molds unless special precautions are taken to sterilize the fluid and prevent access of spores. Many of these molds grow equally well, if not better, upon liquid than upon solid media, and in this respect present marked advantages over the *Mucors* for chemical studies.

Not only are the *Penicillia* and *Aspergilli* widely distributed in nature, but some of the species are of great economic importance. Two species of *Penicillium* are important factors in the cheese industry. The varieties of cheese known as Roquefort, Gorgonzola, and Stilton owe their peculiar flavor to a green *Penicillium* (*P. roqueforti*) which grows in the numerous crevices and air spaces occurring throughout the cheese. Camembert cheese, which is manufactured both in France and in this country, is ripened by another *Penicillium* (*P. camemberti*), which grows upon the surface and secretes enzymes into the curd mass. The rotting of oranges is due principally to *P. italicum* and *P. digitatum*. The mold found upon rotten apples is almost invariably *P. expansum*. In fact, as Thom has pointed out, many of these organisms are so intimately associated with some particular habitat that it is quite easy to obtain a pure culture simply by making a careful transfer. Several species of this genus, especially *P. brevicaulis*, possess the power of transforming arsenical compounds into diethylarsin, which can be recognized even in the minutest traces by its characteristic garlic odor. By virtue of this property *P. brevicaulis* has been employed very successfully as a biological test for arsenic. One-hundredth of a milligram of arsenic can be recognized in this way with certainty, the test being considered fully as delicate as Marsh's test.

The *Aspergilli* are of less economic importance than the *Penicillia*. The strong amylolytic power of some of the species is made use of in oriental countries. *Aspergillus oryzae* is thus extensively used in the preparation of an alcoholic beverage called koji. This is made from

rice by steaming the grains and inoculating with the mold, which soon converts the starch into sugar. At the same time yeast develops, and this transforms the sugar into alcohol and carbon dioxide. Another species, *A. wentii*, is used in Java for making a peculiar condiment or sauce from rice. *A. oryzae*, after it has been cultivated on starch, then extracted with water, and precipitated with alcohol, constitutes the "Taka diastase" of commerce. Certain species of *Aspergillus* are of pathological importance on account of their parasitic habits. Thus *A. fumigatus* has frequently been found growing in the external auditory passage.

ADAPTABILITY TO ARTIFICIAL CULTURE.

Many of the species of these two genera are particularly well adapted for chemical research on account of the readiness with which they thrive upon synthetic culture media. This is undoubtedly the reason why they have been chosen by so many investigators as a basis for chemical experiments. Where a complex medium of uncertain composition, such as bean decoction, meat extract, or the like, has to be used in order to obtain a suitable growth of mold, the changes that take place in the medium can not easily be followed. This difficulty is entirely avoided by the use of a synthetic medium of known composition. In such a medium, where there is a single source of carbon or nitrogen, the carbon or nitrogen assimilation of the mold can be studied by simply varying the carbonaceous or nitrogenous constituent of the medium. As no other molds grow with such facility upon media of this character, these two genera have been used almost exclusively in such experiments.

The readiness with which these molds grow upon fluid media has already been mentioned. When it is desirable to separate the mycelium completely from the substratum, a liquid medium is practically essential. This is particularly true if determinations are to be made upon the mold itself to see what constituents of the media have entered into the actual composition of the fungus. Solid particles adhering to the mycelium would introduce a considerable error. A liquid medium, on the other hand, can be washed out completely without much trouble. The same is equally true if the mold is to be used subsequently in enzym experiments, where qualitative tests would be vitiated by the presence of a protein or carbohydrate introduced in this way. Thus for enzym experiments the *Penicillia* and *Aspergilli* are preeminently adapted.

SOURCES OF CARBON AND NITROGEN.

In the foregoing pages allusion was made to the polyphagous habits of *Penicillium* and *Aspergillus*. A number of investigators have made a careful study of the nutrition of these molds. The presence of pro-

teins or highly complex nitrogenous substances is by no means necessary in the culture medium. The molds are able to synthesize their protoplasm from such simple substances as carbohydrates and nitrates or ammonium salts. While the sources of nitrogen and carbon may be varied within wide limits, the presence of inorganic salts in small amount is necessary. Most of the synthetic media commonly used in cultural work consist, therefore, of a dilute solution of these inorganic salts, to which has been added a carbohydrate, such as ordinary cane sugar, and a nitrogenous body varying in complexity from the nitrate of an alkali metal to a mixture of proteoses and peptones or gelatin. Nitrogen in some combined form is essential, for the molds can not utilize atmospheric nitrogen sufficiently to produce a normal colony. Often a slight growth of mold has been observed in the absence of nitrogenous compounds, but this is to be regarded as due rather to the reserve protein present in the spore itself or to a small amount of nitrogenous substance transferred to the medium during inoculation.

Many of the media in common use are unnecessarily complex. Raulin's fluid has been used perhaps more extensively than any other for cultivating molds. The inorganic constituents of this medium are quite numerous. The nitrogen is supplied in the form of ammonium nitrate and the carbon in the form of cane sugar, while free tartaric acid is added mainly to prevent the growth of bacteria. Raulin found that the yield of mold was diminished to a greater or less extent when any one of the inorganic constituents was omitted from the medium. But as later work has shown, very few of these mineral salts are really necessary. The media introduced by Wehmer and Czapek are much simpler in this respect, yet the fungus growth is, to all appearances, equally good. Some of the metallic elements are needed in such minute traces that the molds can undoubtedly make up any deficiencies of the medium from the glass of the containing vessel.

A great variety of nitrogen compounds may serve as food material for *Penicillium* and *Aspergillus*. A glance at the formulas of some of the well-known fluid culture media will show that nitrates and ammonium salts can furnish all of the nitrogen. With these molds there seems to be no particular advantage in using organic compounds of nitrogen, although the molds can utilize amids, amino acids, peptones, and some heterocycles. In the case of amino acids, according to Emmerling, the amino group must be in the alpha position with respect to the carboxyl, or else attached to alternate carbon atoms. Thus beta-amino acids are unavailable as nutritive material, while gamma-aminobutyric acid yields a good crop of mold. Czapek found that *Aspergillus niger* could not utilize hydrazins, oxims, basic heterocycles, or cyanogen compounds. The writer has

likewise repeatedly failed to obtain any growth of mold upon nitrites, or the salts of hydrazin or hydroxylamin. The mold does have the power of utilizing the nitrogen of certain heterocycles, such as succinimid and nicotinic acid.

The source of carbon can likewise be varied greatly. Emmerling has shown that the molds grow luxuriantly upon a great variety of pentoses and hexoses. According to Reinke the ordinary organic acids, except carbonic, oxalic, and formic, when in combination with bases, are assimilated by *P. glaucum*. Moreover, cyclic compounds, such as parabanic and benzoic acids, can supply the mold with carbon. Diakonow goes still further and states that even formic acid can be utilized by *Penicillium*. Hasselbring tested a number of carbon compounds with respect to their availability as sources of carbon, and found that *P. glaucum* assimilates alcohol, acetic acid, and substances from which the group CH_3COO — is easily derived. According to Czapek the benzene ring is more readily attacked when it bears a number of hydroxyl groups, and such substances as quercite and quinic acid are easily assimilated. A great many of the substances, however, which are recorded in the literature as sources of carbon, yield only a scanty growth, which often fails to fructify. To secure a good thrifty colony, a substance containing one or more asymmetric carbon atoms seems to be necessary, and this is probably the reason why the sugars, from the trioses to the trisaccharids, are sources of carbon par excellence.

Since these two genera of molds, as all other fungi, contain no chlorophyll, they are obviously incapable of carrying out any photosynthesis. They must therefore derive their energy entirely from the chemical reactions which they institute in the culture medium. In other words, the medium must contain sufficient oxidizable substance, or substance capable of undergoing exothermic changes, to render the organism independent of solar energy. The chemical changes must of necessity be quite profound in order to meet these requirements. *Aspergillus niger* is said to have the power of converting one-third its weight of sugar into volatile products in a single day. While it destroys a smaller quantity of sugar than does yeast, it uses up the sugar more completely by combining it with oxygen. Assuming that the final products are carbon dioxide and water, the same amount of energy would be derived from one-twentieth the amount of sugar that the yeast consumes.

CHANGES IN THE CULTURE MEDIUM.

Some of the changes produced in the medium during the development of mold can not fail to be noticed by the most casual observer. For example, the change in color is usually quite pronounced. In some instances this change in color, or rather production of color, is

so striking and characteristic as to form the basis for the specific name of the fungus. Thus *P. purpurogenum* colors the medium a brilliant reddish purple, and *P. chrysogenum* under certain conditions colors it a beautiful golden yellow. Nearly all the species when grown upon a colorless medium containing carbohydrate give rise to a brownish color, due probably to the formation of humus substances.

Other chemical changes, though not so easily detected by the eye, are none the less striking. If the reaction is at first acid to litmus, on account of the presence of free organic acid or acid phosphates, many species of *Penicillium* destroy the acid or else give rise to basic products, leaving the medium neutral or alkaline to litmus, though rarely alkaline to phenolphthalein. Other species produce acid substances and the substratum remains strongly acid to litmus. This seems to be particularly true of some of the *Aspergilli*. The cause of this acidity has been found by Duclaux to be due to the formation of oxalic acid. This is an intermediate product in the oxidation of carbohydrate, and if the mold is removed at the proper time a large yield of oxalic acid may be obtained. Wehmer succeeded in obtaining 80 per cent of the theoretical yield by the incomplete oxidation of cane sugar by means of *A. niger*. In the presence of a calcium salt the oxalic acid is removed from solution as fast as it is formed and can then undergo no further oxidation. Under ordinary conditions, however, the oxalic acid accumulates only so long as an excess of sugar remains, and when this becomes scarce, the oxalic acid in turn is burned up. The *Penicillia*, on the other hand, do not seem to have the power of producing oxalic acid to so considerable an extent; but Wehmer has shown that certain of the species produce citric acid. The citric acid is likewise an intermediate product which finally disappears, and it apparently serves the same purpose as the oxalic acid, namely, that of controlling within certain limits the reaction of the medium.

SEPARATION OF RACEMIC MIXTURES.

In connection with the effect of fungus growth upon the organic constituents of the medium, the peculiar selective power of these organisms is worthy of mention. *P. glaucum* and *A. niger* have been used by a number of investigators for preparing optically active substances from racemic mixtures. This property was first observed in 1860 by Pasteur. He prepared a solution containing the ammonium salt of racemic acid and a small amount of inorganic phosphate and inoculated it with spores of *P. glaucum*. After the mold had grown for a time the solution became levo-rotatory. The dextro acid was found to be completely destroyed, while the levo acid remained intact. Le Bel used the two molds for preparing d-methyl-

propylcarbinol and l-propylglycol from the respective racemic mixtures. His cultures were, however, contaminated with yeasts and bacteria. In 1882 Lewkowitsch prepared d-mandelic acid from the racemic mixture by means of *P. glaucum*. Inactive lactic acid was converted in the same way into the levo form by Linossier. By the same method Schulze succeeded in preparing optically active leucin and glutaminic acid from the inactive mixtures. The amino acid remaining is always the one that does not occur in nature, and Schulze claims that these amino acids can be obtained in the form isomeric with the original substance by racemizing and then subjecting to the action of *P. glaucum*. Buchner notes that *P. glaucum* and *A. niger* grow readily upon the ammonium salts of fumaric, but not on those of maleic acid, and gives us a method of separating isomeric mixtures where the isomerism is due to the presence of a double bond. In 1899 Fischer inoculated a solution containing d-alanin and inorganic salts with *A. niger* and found that 10 per cent of the d-alanin was used up in fifteen days, the remaining solution being levo-gyrate. And still more recently Neuberg and Mayer obtained l-cystin from the inactive mixture by *A. niger*.

Other chemical changes in the medium, especially those of the nature of hydrolytic cleavage, such as the breaking down of proteins, the inversion of disaccharids, and the liberation of ammonia from amids, are due to enzymes; which can be made to act independently of the cells themselves. These changes will be discussed later on under the subject of enzymes.

BEHAVIOR TOWARD ANTISEPTICS.

Before entering upon a discussion of enzymes, however, it might be well to mention very briefly the behavior of these molds toward antiseptics. This problem has been studied by a great number of investigators, and so many observations have been reported that it would be out of the question to give a detailed discussion here. It is well known that molds are far less sensitive to antiseptics than are bacteria. The literature contains many observations of instances where these molds were found growing in solutions of substances known to possess germicidal properties. A table showing the relative toxicity of a number of the well-known antiseptics has been worked out by Malenkowitsch. According to his observations, the salts of the heavy metals are most toxic, while some of the organic antiseptics must be present in considerable amount before growth of mold is inhibited. It appears that those substances are most toxic which act oligodynamically, or those which, like chloroform, toluene, and ether, have a solvent action upon fats and lipoids.

REVIEW OF PREVIOUSLY PUBLISHED WORK ON THE ENZYMES OF *PENICILLIUM* AND *ASPERGILLUS*.

Although the effect of *Penicillium* and *Aspergillus* upon various natural substrata and upon synthetic culture media had been observed for many years, the changes were not ascribed to the action of enzymes. This may be explained upon the ground that the enzymes are for the most part intracellular. Attempts to demonstrate the presence of an enzyme in the substratum after removing the mold would therefore result in failure. The secretion of enzymes into the culture medium does not ordinarily occur to any appreciable extent during the active vegetative period of the mold, but at the time of fructification, and subsequently, some of the enzymes are liberated. Pathological conditions—such as exhaustion of the medium, lack of aeration, or prevention of fructification—have a similar effect. The liberation of the enzyme has been regarded by a number of investigators as due rather to the disintegration of some of the cells than to secretion by active living cells. Any procedure that tends to produce plasmolysis of the cells is therefore accompanied by liberation of the enzymes. Various methods of preparing enzyme solutions from molds are based upon this principle.

THE VARIOUS METHODS OF PREPARING THE ENZYMES.

A method devised by Duclaux and used in a number of his experiments consists in allowing the mold to grow upon Raulin's fluid until spores begin to form, then siphoning off the medium and replacing it several times by distilled water, and then, after the last traces of nutrient material have been washed out, allowing the mycelium to remain in contact with the distilled water for several hours. In this way the cells are made to yield up their enzymes to the water.

Another method, used by Bourquelot, is based upon the destruction of the cell wall by more heroic treatment. It consists in removing the mold at the appropriate time from the culture medium and triturating it in a mortar with sand and chloroform water. The extract thus obtained is filtered and may be used either directly or after precipitation with alcohol.

A third method consists in drying the mold at a low temperature (40° C.) and grinding the resulting product to fine powder. The advantage of this method is that the enzymes in the dry state retain their activity much longer. A solution of the enzymes may be obtained at any time from this powder by simply extracting with water and filtering. These three methods have been employed in all of the earlier work on mold enzymes.

Two other methods introduced by Buchner have been used extensively in preparing yeast enzymes, but have only very recently been

applied to molds. One of these makes use of the hydraulic press, which squeezes out the liquid contents of the cells. The other is the simple dehydration of the organism by means of acetone and ether. The latter method will be described more fully in the experimental part of this paper.

Most of the investigators have examined the enzymes at the time of fructification of the mold. This varies considerably with the individual species. Some of the green *Penicillia* produce spores within three days, while with other *Penicillia* the conidia may not appear until after a week or ten days. There appears to be good reason for preparing the enzymes at this particular stage if the maximum activity is desired. It can be shown experimentally that the amount of intracellular enzyme rapidly diminishes after fructification, and at the same time the enzymes begin to appear in the substratum. The proper time to prepare the intracellular enzymes is, therefore, just before fructification. The explanation given by some authors is that fructification is accompanied by a disintegration of mycelial cells, and the enzymes contained in them are thus set free. Histologists claim, however, that there is no evidence of any disintegration of cells, at least not until after the crop of spores is fully developed. It is not unreasonable to suppose that during the vegetative growth of the organism the enzymes are constantly being produced and stored up within the cells. Then when spore formation begins it is accompanied by increased metabolic activity, and in response to the plant's greater need of easily assimilable nutrients the enzymes are turned loose to act in the medium. Hence to obtain the greatest yield of endoenzymes, they must be caught before they escape as ectoenzymes.

THE PARTICULAR ENZYMES FOUND.

Having briefly mentioned the general methods by which the intracellular enzymes of *Penicillium* and *Aspergillus* may be obtained, we now turn our attention to a consideration of the individual enzymes that have been found in these molds. With our present knowledge of zymology it is impossible to separate individual enzymes from a mixture. The only instance on record where a satisfactory separation has been accomplished is that described by Vines, where he isolated pepsin and erepsin from the mixture occurring naturally in plants. Most of the enzymes, however, are so closely similar in their solubility and behavior toward reagents that attempts to separate them have resulted in failure. But by preparing enzymes from different sources we can obtain preparations that are active toward certain zymolytes and inactive toward others, and in this way our knowledge of the specificity of enzymes has been acquired. When we have to deal with a mixture, therefore, as in the case of mold extracts, the

different types of enzym reactions can be studied separately by testing the activity of the mixture toward different substrata, and from these observations inferences can then be drawn as to the presence of the individual enzym responsible for these reactions.

The enzymic activities ascribed to *Penicillium* and *Aspergillus* species are briefly stated in Wehmer's review. As this author does not, however, give any description of the methods employed by other investigators in their search for enzymes, it was thought advisable to discuss the literature somewhat in detail in this place. The methods and results will be given as described by the authors and with very little comment. Later on it will be shown that the results obtained by the different investigators are not strictly comparable with each other nor with those obtained by the writer, owing to the fact that different species were used. The differences are, however, quantitative rather than qualitative.

PROTEASE.

An examination of the literature will show at once that the proteolytic enzym of *Aspergillus* and *Penicillium* has received more attention from previous investigators than any other single enzym. Yet there are so many conflicting statements that the identity of this enzym is by no means established. Hansen was the first to turn his attention to this subject. He found that when *P. glaucum* was grown upon 6 per cent gelatin the latter was rapidly liquefied. He also made a glycerol extract of the mold just after the liquefaction of the medium had begun and only a few spores had formed. This extract was found to liquefy gelatin in neutral solution more rapidly than in the presence of acid. Attempts to prepare the enzym in the dry state failed. Several years later Wehmer confirmed the observations of Hansen. He found that various species of *Penicillium* and *Aspergillus* liquefy the gelatin of culture media to a considerable distance beyond the colony itself or beyond the reach of the mycelium. The liquefaction was greatest when gelatin was the only source of carbon.

Bourquelot studied the proteolytic enzym of *A. niger* after it had been cultivated on Raulin's fluid. An extract of the mycelium was prepared by triturating with sand and chloroform water. To one portion of this extract a few flocks of fibrin were added, while in another portion were placed a few pieces of coagulated egg white, and both were warmed to 40° C. In both cases there was an unmistakable formation of peptone. The filtered liquid gave only a slight cloudiness with nitric acid, and a characteristic red biuret reaction. Bourquelot concludes, therefore, that the extract contained trypsin. In testing for pepsin he followed the same procedure except that the liquid was made 0.2 per cent acid with hydrochloric acid. There was a disintegration of the fibrin and a slight solvent action upon the

albumin. But the tests for peptone were negative, and the author considers that the presence of pepsin is very doubtful. Bourquelot noticed also that cultures of *A. niger* possess to a limited extent the property of liquefying gelatin.

In 1900 Malfitano made a more detailed study of the protease of *A. niger*. He used Fermi's liquid gelatin method. The method as adapted by Malfitano consists in mixing 10 c. c. of the solution to be tested with 5 c. c. of a warm solution of 20 per cent gelatin containing 0.2 per cent thymol. At room temperature the mixture is solid, but at 35° C. it is liquid. The mixture is left in the thermostat for a given length of time, then cooled down to 15° C. If any digestion has occurred, the solution remains liquid, otherwise it quickly solidifies. By means of this method Malfitano was able to make a number of interesting observations. He found that the enzyme was active only in acid solution. He studied the enzyme in the medium (Raulin's fluid) as well as in the mycelium. In the medium the enzyme showed its greatest activity when the mycelium had reached its maximum growth. The quantity of enzyme was found to be dependent upon the intensity of development of mycelium and upon the degree of maturity. Conditions of aeration or temperature had no direct influence, except in so far as they influenced the growth of mold. He found that when cubes of coagulated egg white or flocks of fibrin were introduced into Raulin's fluid in place of the nitrogen-containing salts they failed to yield any growth of mold. Casein and gelatin furnished an available source of nitrogen, and were still more readily attacked in the presence of other nitrogenous food material.

Malfitano studied also the proteolytic enzyme contained in the mycelium. The enzyme solution was prepared by Bourquelot's method of grinding the mycelium with sand and chloroform water. The activity of the enzyme in the mycelium was found to reach its maximum at the time of spore production, then the cells began to yield up their enzyme to the medium, and the enzyme in the medium increased at the expense of that in the mycelium.

In a second paper Malfitano describes an experiment in which *A. niger* was allowed to develop until the time of sporulation, then the mycelium was removed from the medium and dried at 35° C. An extract of this product digested gelatin readily, and the formation of protogelatinose, deutergelatinose, and gelatin peptone was demonstrated. The extract would not digest Mett's tubes of coagulated egg albumin nor boiled fibrin. Fresh fibrin, on the other hand, was digested in the presence of one-hundredth normal acid, while the fibrin in the control test merely swelled. Milk first curdled and then digested, but the coagulum first formed did not disappear completely. The presence of caseose and peptone was demonstrated in the digested milk.

In the same year Steffens reported some observations on the relation between the formation of proteolytic enzyme and the nutrition of mold. He used four species of *Penicillium* (*P. glaucum*, *P. luteum*, *P. rubrum*, and *P. italicum*) and five of *Aspergillus* (*A. niger*, *A. glaucus*, *A. oryzae*, *A. wentii*, and *A. fumigatus*). These were all grown upon five different media, as follows: Raulin's fluid, starch paste, starch paste and peptone, diluted milk, and egg white and water. After eight days the mold was ground with sand and chloroform water, filtered, and the enzyme precipitated by alcohol. Its digestive action was tried upon egg albumin, casein, fibrin, and "vegetable casein" in the presence of 0.2 per cent hydrochloric acid and 0.2 per cent sodium carbonate. The author used the biuret test to determine whether digestion had taken place. This he considers more delicate than Fermi's gelatin method, for it gave positive results where Fermi's test was negative. Needless to say, he obtained evidence of digestion both in acid and alkaline solution of all the four proteins used. No mention is made of control tests with boiled enzyme or with the enzyme solution alone.

A study of the effects of living molds upon proteins was made by Butkewitsch. He found that when *A. niger* was grown upon "Witte peptone" most of the nitrogen was converted into ammonia, which neutralized the oxalic acid produced simultaneously. Other nitrogenous substances, among which were leucin and tyrosin, were also formed, but in relatively small amount. *P. glaucum*, on the other hand, produced much less ammonia and relatively more amino acids. As no oxalic acid was produced by this organism, the reaction of the medium became alkaline. Butkewitsch claims that the proteolysis is due to the secretion of an enzyme resembling trypsin. This enzyme is contained not only in the mycelium, but also in the culture medium. When the mold is grown upon peptone, more proteolytic enzyme is formed than when the source of nitrogen is ammonium tartrate. The further decomposition of the amino acids into ammonia is an independent process which serves the purpose of maintaining the proper reaction of the medium. It may be influenced in a variety of ways. If calcium carbonate is added to the *Aspergillus* culture, the oxalic acid combines with it and prevents its accumulation in the medium. Consequently there is no need of ammonia, and less is produced. These conditions may be reversed in the case of *Penicillium*. If phosphoric acid is added to the *Penicillium* culture, which normally produces very little ammonia, there is a marked increase in ammonia formed.

A comparison of the results obtained by these authors would indicate that the organisms studied contain a protease that acts readily upon gelatin and less readily upon fibrin and egg albumin. In spite

of conflicting statements regarding the effect upon fibrin and egg albumin, the authors have held to the view that the enzym is of the nature of trypsin.

NUCLEASE.

The effect of cultures and enzym preparations of *A. niger* and *P. glaucum* on thymonucleic acid was studied by Iwanoff. He found that while the sodium salt of thymonucleic acid could not supply the molds with carbon, it furnished a good source of nitrogen and phosphorus. The molds were accordingly grown upon a nucleic acid solution to which had been added cane sugar, magnesium sulphate, and potassium chlorid. After several days the culture fluid was found to give a precipitate of purins with ammoniacal silver nitrate, and a copious precipitate with magnesium mixture. In one experiment which was conducted for thirty-two days the decomposition of the nucleic acid was complete, 70 per cent of the phosphorus appearing as inorganic phosphate and the other 30 per cent entering into the cell structure of the fungus. The author showed by separate experiment that the cleavage could not have been caused by the oxalic acid produced by the mold. To prove conclusively that it was due to an enzym contained in the mycelium, the latter was triturated to a paste with infusorial earth and added to a solution of nucleic acid containing 150 mg. P_2O_5 and chloroform as an antiseptic. In two weeks 7 mg. P_2O_5 and 12.7 mg. nitrogen as purin bases were liberated. A control experiment with boiled enzym gave no purin precipitate. The author claims that this enzym, which he calls nuclease, is entirely distinct from the proteolytic enzym, for the mycelium paste did not liquefy gelatin.

AMIDASE.

An extensive study of the amidase of *A. niger* was made by Shibata in 1904. Previous to that time the power of fungi to liberate ammonia from amids and amins had been observed by Butkewitsch and others, but only in culture media. Shibata now succeeded in hydrolyzing these compounds by means of enzym preparations. *A. niger* was grown upon a medium containing peptone, sugar, and inorganic salts. Two methods were used in preparing the enzym. The mycelium was collected and forced through a sieve, then it was either allowed to dry at ordinary temperature or else treated with acetone and ether according to Albert and Buchner's method for yeast. A portion of the powder thus obtained was mixed with a solution of the substance to be tested, toluene added, and the mixture left in the thermostat for several days. The ammonia was then determined by distillation with magnesium oxid, or in the case of the less stable urea derivatives and acid amids the method of Schlössing was

employed. Urea and acetamid yielded the most ammonia, though appreciable amounts were also obtained with biuret, oxamid, benzamid, asparagin, and alanin. Urethan, guanidin, allantoin, and uric acid gave no ammonia at all. Hippuric acid was decomposed into benzoic acid and glyccoll.

In repeating these experiments with an acetone preparation of yeast Pringsheim obtained negative results. A suspension of yeast, however, readily liberated ammonia from amino compounds. It is evident therefore that the amidase of *Aspergillus niger* is more resistant to acetone and ether than that of yeast.

LIPASE.

The fact that molds can utilize fats as well as carbohydrates was first noticed by Van Tieghem. He found that when a porous body that had previously been soaked in water was immersed in an animal or vegetable oil the surface of this body soon became covered with a growth of mold, the predominating species of which was *P. glaucum*, and, strange to say, the mold thrived and produced normal fructification, although apparently excluded from air by the layer of oil. At first there seemed to be no change in the oil, but after a time a white crystalline mat began to form where the oil was in contact with the mycelium. The crystals were needle-shaped, and were at first very small, but they increased in size until they attained a length of 1 to 2 mm. The author states that these crystals consisted of fatty acids, produced by hydrolysis of the fat.

Gérard cultivated *P. glaucum* upon Raulin's fluid to which a little monobutyryl had been added, and noticed that there was a liberation of butyric acid. The fat-splitting power of this organism was shown by him to be due to an enzyme contained in the mycelium. An aqueous extract of the mold had the power of hydrolyzing monobutyryl. To 100 c. c. of a 2 per cent monobutyryl solution he added 30 c. c. of the mold extract. The number of cubic centimeters of decinormal alkali that it required to neutralize this solution increased from 0.4 in the control to 2.5 in the digestion experiment in six days. This represents less than 3 per cent of the theoretical yield of butyric acid, and the lipolytic action must therefore have been quite feeble.

Camus confirmed the observation of Gérard, and found also some slight evidence of lipolytic action in *A. niger*. Cultures of *P. glaucum* upon monobutyryl grew poorly, and the lipolytic action was not very marked. The medium did, however, increase in acidity, and this the author attributes to the liberation of butyric acid. In the case of *A. niger* the lipolytic action was still more feeble.

Garnier grew *A. niger* upon a modified Raulin's fluid at 35° C., then filtered off the mycelium and allowed it to act upon a solution of monobutyryl in the presence of chloroform. A short time after

inoculation, just as the spores began to appear, there was evidence of a small amount of lipolytic enzym. The amount of this enzym quickly diminished and remained very small for about eleven days. At the end of that time the activity began to increase, without, however, becoming very marked. It reached its maximum on the fifteenth day, after which it remained practically constant for about two months. The same author studied the lipase of *A. fumigatus* and found it to be very feeble. Two days after inoculation a slight lipolytic action was observed, but this disappeared at the time of fructification. At the end of twelve days, when the surface of the colony was completely covered with spores, the lipase reappeared, but only in small amount. *A. flavus* and *A. glaucus* behaved in the same way, though the latter yielded somewhat more lipase.

The action of *P. glaucum* on butter fat was studied by Laxa. He noticed that butter inoculated with this organism developed considerable acidity. By extracting the mycelium obtained by inoculating this organism on media containing asparagin and lactic acid he obtained an enzym which hydrolyzed monobutyryl.

In more recent years Deleano has studied the lipase of several molds. He cultivated *P. glaucum*, *A. niger*, and *A. flavus* on Raulin's fluid for seventeen days. The mycelium was then filtered off and dried at 37° C. The medium upon which the molds had grown showed no lipolytic power. Various solutions were used in extracting the dried mold. Physiological saline with 5 per cent glycerol, 1 per cent sodium hydroxid, and 50 per cent glycerol extracted no lipase. When the powder was extracted for two days in the thermostat with 1 per cent sodium phosphate and 0.5 per cent alcohol some lipase was obtained. The extract made from *A. niger* in this way acted strongly upon tributyrin, feebly upon monobutyryl, while it had no action at all upon ethyl butyrate. *A. flavus* showed a feeble action upon tributyrin and ethyl butyrate and none upon monobutyryl. *P. glaucum* failed to hydrolyze any of the three esters. From these experiments the author suggests the possibility of specific enzymes for different esters.

EMULSIN.

A glucosid-splitting enzym was observed almost simultaneously in 1893 by Gérard and by Bourquelot in two different molds. Gérard prepared an extract from the mycelium of *P. glaucum* by trituration with sand, and precipitated the enzymes from this extract by the addition of alcohol. After dissolving and reprecipitating, the product was washed with ether and dried in vacuo. One-hundredth of a gram of this preparation hydrolyzed 10 c. c. of a 1 per cent amygdalin solution completely into glucose, benzaldehyde, and hydrocyanic

acid in twenty-four hours. Salicin was likewise completely hydrolyzed in the same length of time.

Bourquelot prepared his enzyme from *A. niger* by growing the organism on Raulin's medium until the time of fructification, then floating the mycelium on distilled water for several days. The solution obtained in this way when allowed to act upon amygdalin gave the characteristic odor of benzaldehyde within an hour. Bourquelot and Hérissé found that not only could amygdalin be hydrolyzed, but also salicin, coniferin, arbutin, esculin, populin, and phlorhizin. On the other hand, some of the less soluble glucosids, such as solanin, hesperidin, convallamarin, convolvulin, digitalin, and jalapin remained unaltered.

A comparison of the emulsin of *A. niger* with that of almonds was made by Hérissé. He tested the action of the enzyme prepared from both sources on a number of glucosids. While the enzyme from *A. niger* was found to decompose populin and phlorhizin, the emulsin of almonds had no effect upon these two substances. There was also a decided difference in the rate at which the two enzymes decomposed other glucosids. The enzyme of *Aspergillus* acted more energetically upon arbutin than upon any other glucosid, while the reverse was found to be the case with the emulsin of almonds. Further investigation of the glucosid-splitting power of *A. niger* showed that the formation of this enzyme could be entirely suppressed by an excess of ammonium nitrate in the medium, but upon replacing this solution by water an abundance of emulsin was formed. The author believes that a condition of starvation may result in the formation of enzymes that were previously absent.

The glucosid-splitting power of the same organism as well as that of *A. glaucus* and *P. glaucum* was investigated by Puriewitsch. This author experimented with the living mycelium. The mold was grown upon Raulin's fluid, then the liquid removed and replaced two or three times by distilled water and finally by a solution of the glucosid. When salicin was used, a cleavage was evident in fifteen to twenty hours. The salicin decomposed with the formation of saligenin which gives a blue color with ferric chlorid. The intensity of the color increased with the time during which the mold was allowed to act. No glucose, however, could be demonstrated by Fehling's solution, and it was evidently used up by the mold as fast as it was formed. Similar results were obtained with helicin, arbutin, coniferin, esculin, phlorhizin, and hesperidin, though of these only the first two give products that can easily be detected by odor or color reactions. Amygdalin behaved quite differently according as the living mycelium or an extract was used. Upon solutions of amygdalin the mold grew well, but no odor of benzaldehyde and no hydrocyanic acid could be detected. The nitrogen was liberated in

the form of ammonium salts. When, however, an extract of the mycelium was used, the amygdalin was rapidly decomposed into benzaldehyde, hydrocyanic acid, and glucose. The selective action of the mold is quite apparent from these experiments. The organism uses up the glucose and leaves the benzene derivative intact until the former is exhausted; then the latter is utilized also. Moreover, in the presence of a large amount of glucose or cane sugar the glucosid itself is not attacked.

The stereochemical configuration of the glucosid molecule is of importance in determining the effect of the enzym. Pottevin found that a solution of *A. niger* which had been cultivated on Raulin's fluid acted on amygdalin and on beta-d-glucosids, but not on the two methyl-galactosids nor on lactose. When, however, lactose or methyl-d-galactosid was added to the culture medium, an enzym capable of hydrolyzing them was developed.

Brunstein again took up the study of the effect of certain fungi on media containing glucosids. Among the organisms cultivated were *A. niger*, *A. oryzae*, *A. wentii*, *A. glaucus*, and *P. glaucum*. These were grown upon media consisting of Raulin's fluid and peptone, and after the mold had reached maturity the culture fluid was replaced by a solution of glucosid. The relative nutritive value of the different glucosids was determined by noting the increase in weight of the mycelium. Coniferin was most readily utilized, while arbutin, helicin, and salicin had a toxic effect. The latter was due to the liberation of the toxic substances hydroquinone, salicylaldehyde, and salicylalcohol from these glucosids. Certain of the organisms overcame the toxic action of salicin by oxidizing the salicylalcohol liberated to aldehyde and then to salicylic acid.

"TANNASE."

The power possessed by *Aspergillus* and *Penicillium* to hydrolyze tannin was observed as early as 1867 by Van Tieghem. This author noticed that a solution of tannin does not undergo change if it is kept saturated with carbon dioxide or is rendered sterile by heating. But when the solution is left exposed to the air, molds make their appearance and decompose the tannin into gallic acid which crystallizes out. Inoculation of tannin solutions with pure cultures of *P. glaucum* and *A. niger* gave the same phenomenon. As his attempts to decompose tannin by an extract of the mycelium were unsuccessful, he concluded that the decomposition of tannin with the formation of gallic acid was a true fermentation in Pasteur's sense, and he called it "gallic fermentation."

In 1900 Fernbach again took up the subject of gallic fermentation. He grew *A. niger* on Raulin's fluid in which the sugar had been

replaced by tannin, and found that the tannin was slowly decomposed with the formation of gallic acid. An enzyme preparation obtained from this culture was more active and hydrolyzed a 10 per cent solution of tannin at 50° C., so that on cooling crystals of gallic acid separated out. The mold extract filtered through a Chamberland filter and added to a sterile solution of tannin gave the same results.

Almost simultaneously with Fernbach's work, Pottevin obtained an enzyme in the same way which decomposed commercial tannin into gallic acid and 12 to 15 per cent glucose. No tannase was formed unless tannin had been added to the medium on which the organism was grown.

The "tannin" used in these experiments must have been a glucosid of some sort.

ENZYMES ACTING ON CARBOHYDRATES.

It is generally understood that the complex polysaccharids, as well as the more simple disaccharids and trisaccharids, are not directly assimilable. They must first undergo a process of cleavage by which they are transformed into monosaccharids, and this is accomplished by various enzymes, each of which is specific for a particular carbohydrate. The specificity of these carbohydrate-splitting enzymes is very marked, and it is possible to prepare enzymes which act vigorously upon a single carbohydrate and no other. No one, however, has succeeded in separating mixtures of these enzymes, and where a given preparation acts upon a number of complex sugars it must be assumed that the various activities are due to individual specific enzymes. The significance of this will at once be seen in a study of the lower fungi. As the molds can utilize a great variety of complex sugars, the enzymes which decompose these sugars must all be present together, unless we are to assume that the organism possesses a certain power of adaptation by which an enzyme is formed only when it is needed for the utilization of that particular substratum. With this view in mind we now turn our attention to a review of the various sugar-splitting enzymes found in *Penicillium* and *Aspergillus*.

AMYLASE.

The formation of amylase by *Aspergillus* and *Penicillium* seems to be subject to variations depending upon the species and upon the nutrient medium on which the organism is cultivated. According to Duclaux, *A. glaucus* when cultivated on solutions containing calcium lactate as the only source of carbon produces amylase but no protease nor sucrase. On the other hand, when cane sugar served

as the source of carbon, sucrase was formed but no amylase. *P. glaucum* produced sucrase on both media, but the amylase was much weaker. With glycerol as the source of carbon a feeble amylase was obtained. Duclaux states also that *A. niger* does not germinate on starch, but is able to utilize starch if the mold is first cultivated on a sugar solution and the mycelium transferred to an amylaceous medium. *A. oryzae* germinates on sugar solutions, but thrives less well upon such media than upon starch. According to Petit, *P. glaucum* and *A. niger* grow upon solutions of dextrin. The medium acquires a reducing power and maltose and dextrose may be demonstrated.

In general it may be said that the *Penicillia* secrete less amylase and are consequently less well adapted to grow upon starch-containing media than the *Aspergilli*. Hansen states that his *Penicillium* which had been cultivated on gelatin had no action at all on starch paste.

With an extract of the mycelium of *A. niger*, Bourquelot tested the amylolytic action on a 0.5 per cent paste of potato starch. After thirty hours the mixture gave no color with iodine, and with Fehling's solution a reduction was obtained corresponding to 92 per cent of the theoretical amount of glucose.

The organism which produces amylase in greatest amount is *A. oryzae*. This is the source of the commercial "Taka diastase." A description of the preparation and properties of the enzyme is given in Takamine's paper. According to this author the organism is grown on wheat bran, the mycelium extracted with water, and the extract precipitated by alcohol and dried. The resulting product is more active than malt diastase, and has the power of hydrolyzing 100 parts of starch in ten minutes.

An interesting study of the effect of the culture medium upon the formation of amylase was made by Katz. He found that when the medium contained 0.25 per cent starch *P. glaucum* hydrolyzed the starch, so that in two days the iodine test was negative. The growth on starch was not, however, luxuriant. If the medium contained 1.5 per cent cane sugar in addition to the starch the growth was more vigorous, but the disappearance of the starch was considerably delayed. Fifteen per cent sucrose or 2 per cent dextrose completely suppressed the production of amylase. This was not due to any effect upon the enzyme itself, for the amylase acts readily on starch when sugar is present in this concentration. An extract of the mycelium from a culture that had been obtained with 0.25 per cent starch and 2 per cent sucrose had no effect at all upon starch. Maltose and lactose did not suppress the amylase, even if present to the extent of 10 per cent. Erythro-dextrin had no effect, nor had quinic

acid, glycerol, tartaric acid, or meat extract when added to the medium. The addition of peptone caused the inhibitory effect of the sugar to vanish.

The formation of amylase by *A. niger* was less susceptible to these inhibitory influences than *P. glaucum*. On solutions containing only starch, the growth was slow and the amylase rather feeble. The disappearance of the starch was accelerated by the addition of 1.5 per cent sucrose. Further addition of sucrose delayed the disappearance of the starch, but a concentration of 30 per cent did not suppress it. The mycelium yielded more amylase when the organism was grown with starch than it did in the absence of starch. From these experiments the author concludes that molds have the power of regulating the production of amylase according to the nutritive requirements, and that this power of regulation is more highly developed in *P. glaucum* than in *A. niger*.

INULASE.

Before the enzyme inulase was discovered, Bourquelot had noticed that *A. niger* grew on a solution containing inulin as the only source of carbon as well as it did on cane sugar. Several years after the publication of the paper by Green,^a Bourquelot again took up the subject of the cleavage of inulin by *A. niger*. An enzyme preparation of this organism obtained by Duclaux's method was found to act readily upon solutions of inulin. A 1.32 per cent solution of inulin was converted in eighty-four hours at 17° C. into 1.40 per cent reducing sugar, while the rotation increased from -1.06 to -2.53. This represents complete hydrolysis. He states that yeast does not ordinarily ferment inulin solutions, but in the presence of a little inulase from *A. niger* the fermentation goes on very rapidly.

A more extended research into the inulase of *A. niger* and *P. glaucum* was made in 1903 by Dean. This author obtained the enzyme by cultivating these organisms for a week on media containing inulin, filtering off the mycelium and immersing it for five minutes in a mixture of absolute alcohol and ether. The mycelium was obtained, on drying, in the form of a grayish powder. This powder readily hydrolyzed solutions of inulin, but no inulase could be detected in the culture fluid upon which the organisms had grown. The enzyme was very sensitive to acids and alkalies. It was destroyed by sulphuric acid or potassium hydroxid in concentrations of 0.01 normal. The optimum temperature was determined as 55° C. The *Aspergillus* powder possessed a rather stronger inulin-splitting power than the *Penicillium* powder.

^a Green, J. R. On the germination of the tuber of the Jerusalem artichoke (*Helianthus tuberosus*). Annals of Botany, vol. 1, p. 233. Oxford, 1887-1888.

Saiki used a preparation of inulase obtained in this way from *A. niger*. By injecting it into the blood circulation of a rabbit he was able to prepare a serum containing antiinulase and antisucrase.

RAFFINASE.

Scheibler's discovery of a raffinose-splitting enzym in yeast led Bourquelot in 1896 to search for this enzym in *A. niger*. He mixed 50 c. c. of a 4 per cent raffinose solution with an equal volume of the culture fluid upon which this organism had grown. In four days the rotation of the mixture had decreased from 4.8 to 4.0, and a reduction of Fehling's solution obtained corresponding to 0.66 gram sugar. This represents one-third the amount yielded when raffinose is completely hydrolyzed. Bourquelot is cautious, however, about making any inferences as to whether the hydrolysis had gone beyond the formation of levulose and melibiose and decomposed the latter into dextrose and galactose.

Three years later Gillot studied raffinose with reference to its availability as a carbohydrate food for *A. niger*. He prepared a culture medium which consisted of Raulin's fluid with raffinose in place of the sucrose. As the free tartaric acid in this medium was found to hydrolyze some of the raffinose at the temperature of the autoclave, the sterilization was effected by filtering through a Chamberland filter. When this medium was inoculated with *A. niger* a colony developed and produced normal fructification. Examination of the medium every twenty-four hours showed that the reducing sugar formed rapidly increased in amount, while the amount of unchanged raffinose diminished. Oxalic acid was produced just as when cane sugar furnished the carbon. The complete disappearance of the carbohydrate the author interprets as evidence of the presence of a second enzym, "melibiase," which breaks up the melibiose first formed into dextrose and galactose.

The hydrolysis and utilization of raffinose by *P. glaucum* was next investigated by the same author. In the first series of experiments the culture medium was prepared as before. *P. glaucum* inverted the raffinose completely in four days, though some of the reducing sugar remained until the twelfth day. To prove that the hydrolysis was really due to an enzym, the mycelium was extracted by the method of Duclaux and the extract allowed to act upon raffinose. In twenty-six days the rotation had decreased from 8.8 to 6.2 and 0.324 gram glucose was formed, corresponding to 22 per cent hydrolysis. Instead of using an antiseptic in these experiments the author heated the mixture every day for two hours at 55° C. He admits the inadequacy of this method as a means of sterilization, but states that in this case it was apparently effective.

SUCRASE.

As early as 1858 Béchamp found that solutions of cane sugar containing small amounts of inorganic salts became levo-rotatory on standing, while at the same time molds developed. A solution of pure sucrose in distilled water did not undergo change, and the solution containing salts likewise remained unchanged when a little mercuric chlorid or creosote was added. The molds were not identified but were described merely as green, yellow, or white, and the mycelium as floating or submerged. There can be little doubt, however, that some of the molds at least were *Penicillia* or *Aspergilli*.

Twenty years later Gayon cultivated *P. glaucum* and *A. niger* on solutions containing cane sugar and noticed that the sugar rapidly became inverted. He noticed also an important distinction between these molds and some of the *Mucors*, the latter not having the power to invert cane sugar. Several years later Duclaux in studying the food value of different substances for *A. niger* likewise noted that sucrose in the medium became inverted.

The presence of an intracellular enzym responsible for the inversion of the sugar was definitely established by the experiments of Bourquelot. In his paper on maltose this author states that an aqueous extract of the mycelium of *A. niger* hydrolyzes cane sugar as well as maltose, and that the extract loses its activity on filtering through porous clay. *Penicillium* likewise yielded a mixture of enzymes, but in this case the action upon sucrose was much more energetic than that upon maltose or starch paste. In a later paper the same author showed that this enzym solution added to an equal volume of a 2.5 per cent sucrose solution gave the theoretical yield of invert sugar in twenty-four hours.

Fernbach made a detailed study of the sucrase of *A. niger*. He prepared a series of flasks, each containing the same amount of Raulin's medium, inoculated them as nearly as possible with the same number of spores, and allowed the organism to grow at 35° C. At different stages of growth the mycelium was filtered off and the filtrate made up to a definite volume, then the invert sugar was determined. In three days the inversion was nearly complete and a large proportion of the sugar had disappeared, while in four days not a trace of sugar was left. Although in two days the mold had reached half its maximum weight, no inverting enzym was found in the medium. But as soon as the medium became deficient in sugar the liberation of enzym began, and in eight days there was six times as much enzym in the medium as there was in three days. The diffusion of sucrose from the cells may be hastened by the exclusion of oxygen and consequent prevention of fructification. The author holds, therefore, that there is no genetic relationship between the

formation of spores and the appearance of sucrose in the medium. This view leads to the assumption that the inversion of sugar is normally an intracellular process. Although the medium itself shows no marked sucroclastic action until fructification has begun or the liberation of enzym has been induced by pathological conditions, the mycelium contains a very active sucrase from the start. Its activity gradually increases, and as the sugar becomes exhausted the greater part of the intracellular enzym is set free and appears in the medium.

According to Fernbach, the sucrase of *A. niger* is most active in an acid medium. He constructed a table showing the amounts of different acids that must be present to produce equal effects. This table was interpreted by Kanitz, who showed that the effect of the acid was determined entirely by the concentration of hydrogen ions. The optimum concentration of hydrogen ions for the sucrase of *A. niger* is from 3×10^{-5} to 3×10^{-6} normal.

MALTASE.

In his classic paper on the physiological properties of maltose Bourquelot describes a series of experiments which demonstrate the presence of a maltose-splitting enzym in *A. niger* and *P. glaucum*. He cultivated the organisms on Raulin's fluid, and when they had reached maturity he siphoned off the culture fluid and replaced it first by water and then by a solution of maltose. By noting the decrease in rotation and determining the reducing power with Fehling's solution he was able to prove that most of the maltose became converted into dextrose. Not only the living mycelium, but also an enzym obtained by precipitating an extract of the mycelium by alcohol had the power of transforming maltose into dextrose. The solution of maltase was found to lose its activity considerably when passed through a porous clay filter. *A. niger* not only continued to grow when the medium was replaced by a solution of maltose and inorganic salts, but the organism germinated readily when such a solution was inoculated with spores. The maltase of *P. glaucum* was not as active as the sucrase present in the same extract.

Bourquelot's experiments were performed without the use of an antiseptic. Ten years later Hérissé prepared a maltase from *A. niger* by floating the mycelium on distilled water. The action of the enzym was tested by the polariscope, and it was found that the addition of chloroform had no inhibitory effect.

LACTASE.

Bourquelot and Hérissé failed to find any evidence of lactase in *A. niger*. Neither the medium nor the mycelium from cultures of this organism on ordinary Raulin's fluid had any effect on milk sugar. Only one instance is on record of the occurrence of lactase in these

lower fungi. Pottevin confirmed Bourquelot's observation that *A. niger* when cultivated on Raulin's fluid produced no lactase. But when the culture fluid was replaced by lactose and inorganic salts after the organism had begun to grow, the mold continued to develop and a lactose-splitting enzyme was found in the mycelium. The same was true of methyl-d-galactosid. This glucosid was not attacked by the mycelium extract unless the substance had been present in the culture fluid. The apparent power of this organism to respond to the nutritional needs by producing new enzymes would be very striking if the author had demonstrated conclusively that all traces of lactase were absent from ordinary cultures.

TREHALASE.

The disaccharid trehalose was found in *A. niger* by Bourquelot, and its wide distribution in fungi was subsequently demonstrated by him. This substance is evidently a reserve carbohydrate stored up in the fungi, and before it can be utilized it must first be hydrolyzed into glucose. Accordingly Bourquelot examined the enzyme preparation obtained from *A. niger* to see if it was capable of hydrolyzing trehalose. On adding the enzyme solution to a solution of trehalose, the rotation decreased in a few hours, and the decrease continued until the sixth day, when the hydrolysis was complete, as was shown by the fact that the theoretical yield of glucose was obtained. According to Bourquelot, trehalase may be distinguished from maltase by the much lower temperature at which it is destroyed. Trehalase loses its activity at 64° C., while maltase remains active until a temperature of 74° C. is reached.

Theoretically, trehalose should be the food par excellence for this organism, as it is stored up for that purpose. But Bourquelot has shown that spores of *A. niger* germinate very slowly on solutions of trehalose. This fact he explains on the ground that only those enzymes are produced during germination which act on the food material contained within the spore. Later on another category of enzymes is produced, by the aid of which the organism can utilize the substances which it has stored up during a previous period of growth.

GENTIANASE AND GENTIOBIASE.

Gentianose, like raffinose, is a trisaccharid, and according to Bourquelot would require two distinct enzymes for its complete hydrolysis. Top yeast accomplishes the first stage of the reaction but carries it no further. Emulsin of almonds then acts on the disaccharid formed and completes the hydrolysis. Bourquelot found that *A. niger*, when grown upon Raulin's fluid produces both enzymes, as a complete hydrolysis of the gentianose occurred, yielding three molecules of hexose.

MELIZITASE.

Melizitose is another trisaccharid that would require two enzymes for its complete hydrolysis. The first stage is the cleavage into dextrose and turanose. The latter may then be still further decomposed by the action of dilute acids into two molecules of dextrose. Bourquelot and Hérissé found that only the first stage of this hydrolysis can be accomplished by *A. niger*. A 2.5 per cent melizitose solution mixed with an equal volume of the *Aspergillus* extract and allowed to digest for four days gave a decrease in rotation and a reduction of Fehling's solution, but the hydrolysis was not complete.

OTHER ENZYMS FOUND.

ZYMASE.

Among the molds there are transitional forms varying from the strictly aerobic type to those which readily adapt themselves to anaerobic conditions. *Penicillium* and *Aspergillus* belong to the former class, but even here we have quantitative differences. According to Duclaux, *Aspergillus* differs from *Penicillium* in that it can support a lack of oxygen for a longer time and with less injurious effects. It can even adapt itself for a certain length of time to an almost total deprivation of oxygen. When the organism is cultivated under partially anaerobic conditions, a small amount of alcohol may be detected in the medium. In the case of *P. glaucum* the amount of alcohol thus formed rarely exceeds one-thousandth of the total volume of the medium. This rapidly disappears again when access of air is restored.

The most successful experiments along this line are those of Kostytshew. This author first cultivated *A. niger* on a modified Raulin medium in which the cane sugar was replaced by dextrose. The rate of production of carbon dioxid was studied both with air access and after the air had been replaced by nitrogen. A marked diminution in the formation of carbon dioxid occurred after excluding the air. In a second series of experiments a culture of this organism three days old was sunk to the bottom of the flask by means of sterile glass beads. The air was then replaced by nitrogen and the flask sealed. After fourteen days in the thermostat the culture fluid was analyzed. The dextrose had decreased from 10.3296 grams to 10.0043 grams, while 0.1563 grams carbon dioxid and 0.1420 grams alcohol had been formed. The ratio between these two products is practically the same as that in the fermentation of sugar by yeast. The loss of sugar not represented by the carbon dioxid and alcohol the author ascribes to the formation of oxalic acid. From these experiments it is clear that the anaerobic respira-

tion of *A. niger* takes on the character of an alcoholic fermentation when the mycelium is completely submerged. When the surface of the culture is exposed to an inert gas, the amount of carbon dioxid formed is insignificant.

Shortly after the publication of Kostytschew's paper, Junitzky proved that the anaerobic formation of carbon dioxid and alcohol by *A. niger* was due to an enzym closely resembling if not identical with the zymase of yeast. He grew the mold on Raulin's fluid until fructification began, then he ground the mycelium with sand and pressed out the juice in a Buchner press with 300 atmospheres. A solution of glucose was added to this juice, and the amount of carbon dioxid and alcohol was determined after twenty-four hours. Although only a small percentage of the sugar was fermented and a large amount of enzym had to be used, the presence of a zymase was demonstrated. This zymase was formed by the mold during its ordinary aerobic vegetative processes.

OXIDASE.

Molds do not produce the characteristic oxidizing enzymes to anything like the extent to which such enzymes are found in many of the higher fungi. Bourquelot, who studied the oxidase and laccase of mushrooms, as well as many enzymes of *A. niger* and *P. glaucum*, reports no observation whatever of the occurrence of any oxidizing enzymes in these lower fungi. Shibata, in his study of the amidases, failed to find any tyrosinase in *A. niger*. Yet it has long been known that the common molds grow normally only in the presence of oxygen and that the medium must contain an oxidizable substance. As the ultimate products are principally water and carbon dioxid, we must assume that an oxidation takes place.

Herzog and Meier studied the effect of cultures of *P. glaucum* upon various hydroxy acids in the medium. Cultures were made upon diluted beer wort, and when the rate of production of carbon dioxid had become constant they added a solution of the hydroxy acid and determined the increased output of carbon dioxid as well as the amount of acid consumed. A fairly constant ratio was found between the increase in carbon dioxid and the disappearance of the acid. Lactic, tartaric, malic, mandelic, and beta-hydroxybutyric acids were readily oxidized, while glycolic, citric, pyroracemic, and hydroxyisobutyric acids showed no appreciable oxidation. The presence of an asymmetric carbon atom is therefore necessary. On killing the mold with acetone or methyl alcohol and quickly drying, the authors obtained a powder which likewise liberated carbon dioxid from hydroxy acids.

In a second paper the same authors showed that the optical antipodes of hydroxy acids are oxidized at different rates. This explains

the separation of racemic mixtures by cultures of fungi, first observed by Pasteur. An enzyme prepared by treating the mycelium with liquid air was found to liberate carbon dioxid from lactic acid. The authors call the enzyme "acidoxydase."

Part of this carbon dioxid undoubtedly comes from the carboxyl group of the acid. In the case of cinnamic acid this type of reaction has been demonstrated. Oliviero showed that *A. niger* and *P. glaucum* transform cinnamic acid into styrol. The organisms were grown on Raulin's fluid until mature, then shaken violently to liberate the enzyme and filtered through a Chamberland filter. On the addition of small quantities of sodium cinnamate the characteristic odor of styrol was noticed. Herzog and Ripke confirmed this observation by growing *P. glaucum* upon a large amount of beer wort to which ammonium cinnamate had been added. In three days the characteristic odor of styrol was noticed, and the substance was isolated and identified.

SUMMARY OF THE LITERATURE.

A considerable number of enzymes have been found by previous investigators in species of *Penicillium* and *Aspergillus*. Those which have been shown to act in the absence of living cells are: Protease, nuclease, lipase, emulsin, amylase, inulase, raffinase, sucrase, maltase, trehalase, gentianase, melizitase, amidase, zymase, and oxidase. Some of these occur in very active form; others are more difficult to demonstrate on account of the small amount present. Lactase is said to occur only when lactose is the source of carbon in the culture medium. The nature of the protease has not been established, though most of the authors consider it as closely resembling trypsin.

EXPERIMENTAL WORK.

CHEMICAL DIFFERENCES IN SPECIES OF *PENICILLIUM*.

The résumé of literature to which the preceding pages have been devoted comprises both the intracellular and extracellular enzymes of *Aspergillus* and *Penicillium*. It will be seen that with very few exceptions previous work has been confined to two species—*A. niger* and *P. glaucum*. This is particularly true of those investigations which deal primarily with the intracellular enzymes. Cultures of these organisms are easy to obtain, as they make their appearance spontaneously upon almost any kind of substratum that contains a carbohydrate. Many of the cultures for enzyme experiments were undoubtedly obtained in this way; for some authors admit having made transfers from media that had accidentally become contaminated with these molds, and it is of course impossible to say with what care the organisms were identified. The fact that binomial nomenclature

is used leaves it to be inferred that the cultural aspects and morphology were found to correspond with the original description. Assuming, then, that this agreement was found, the results are comparable provided the original description is sufficiently accurate to include only a single species. As far as *A. niger* is concerned this is probably true. Van Tieghem's description of this organism is accurate and minute, and moreover there are very few organisms which might possibly be mistaken for *A. niger*. On the other hand, Link's description of *P. glaucum* is so vague as to include all green *Penicillia*. In fact the designation "*P. glaucum*" has almost come to be synonymous with "green mold."

The question now arises, do all species of *Penicillium* show the same physiological and chemical characteristics? A detailed discussion of these fungi from the standpoint of taxonomy would of course be out of place in this paper. Suffice it to say that there are a number of green *Penicillia*, all of which answer to Link's description of *P. glaucum*, but which show marked chemical differences under definite conditions. In a monograph by Dr. Charles Thom^a some half dozen commonly occurring green *Penicillia* are described. Cultural studies of these organisms show that some liquefy gelatin and some do not; some secrete a red or yellow dye into the medium while others do not; and some give a characteristic odor which others fail to produce. Morphological differences are likewise to be found. It is therefore not improbable that differences in enzymatic activity may be equally pronounced. While there is some controversy as to whether it is justifiable to distinguish species on purely physiological grounds, yet in chemical experiments these chemical differences should be taken into account. Many of the conflicting statements in the literature, especially those concerning the proteolytic enzymes, may be explained on the assumption that different organisms were used under the same name. If species so closely related show decided chemical differences, still greater variations may be expected where morphological differences are more pronounced.

THE ORGANISM SELECTED, *PENICILLIUM CAMEMBERTI*.

In selecting a *Penicillium* for enzym experiments the writer has endeavored to use one whose identity is fully established, and to obtain pure cultures from a reliable source. For a number of reasons *P. camemberti* was considered particularly well adapted for these experiments. The cultures were obtained from Dr. Charles Thom, of the United States Department of Agriculture, by whom the fungus was first described. The organism in question is of considerable

^a Thom, Charles. Cultural studies of *Penicillium*. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 118. 1910.

economic importance because of its association with the cheese industry. It is the principal factor in the ripening of the well-known Camembert type of soft cheese. The manner in which it produces the ripening will be discussed in connection with the proteolytic enzym. So intimately is this mold associated with Camembert cheese and so readily is it recognized that it is not a difficult matter to obtain a pure culture by simply making a transfer from a Camembert cheese purchased in the market. This mold possesses the additional advantage of thriving admirably under artificial conditions and producing a dense mycelium rich in enzymes.

CHOICE OF A CULTURE MEDIUM.

Having secured the organism in pure culture, a number of experiments were made in order to determine what medium was most suitable for propagating it. Obviously, if the mycelium of the mold is to be prepared in the form of a dry powder as free as possible from nutrient material, a solid medium such as gelatin or agar is undesirable. All of the fluid media described by Raulin, Wehmer, Czapek, Dean, and a host of others consist essentially of a carbohydrate solution to which a few inorganic salts have been added. Raulin's fluid is particularly well adapted to *A. niger*, but it has been used a great deal for other species also.

The choice of a medium for *P. camemberti* was therefore carefully considered at the outset. Inoculations were made upon most of the media described in the literature, and while the organism seemed to thrive upon all of them, the media which gave the most luxuriant growth were those of Raulin and Czapek, sodium nitrate being added to the latter as the source of nitrogen. Where cultural studies are to be made to determine the effect of nutrition on the secretion of enzymes other carbohydrates may be substituted for the cane sugar. For such experiments Raulin's fluid is objectionable, as it contains enough free acid to decompose some of the more easily hydrolyzable carbohydrates during sterilization. In Czapek's fluid the acidity is due entirely to acid phosphates, and experiments showed that raffinose, inulin, etc., could be sterilized in this medium without undergoing decomposition. This medium was therefore adopted for the sake of uniformity. Its composition is as follows:

Water.....	1,000.0
Magnesium sulphate (cryst.).....	.5
Potassium acid phosphate.....	1.0
Potassium chlorid.....	.5
Ferrous sulphate.....	.01
Sodium nitrate.....	2.0
Cane sugar.....	30.0

METHODS OF CULTURE.

The above-described medium was placed in a large number of Erlenmeyer flasks to the depth of about 3 cm. The mouth of each flask was then closed with a cotton plug and the flasks were sterilized in an autoclave at 110° C. for half an hour. Occasionally a slight cloudiness appeared in the medium after sterilization, due probably to the precipitation of magnesium phosphate, but this disappeared after the mold had begun to grow. Inoculations were made from a pure culture by means of a sterile platinum wire. The cultures were allowed to develop in diffuse daylight at laboratory temperature. In about three days white specks were seen floating on the surface of the fluid, and these rapidly spread until the whole surface became covered with a dense white mycelium. The mycelium continued to develop and increase in thickness until about the tenth day, when the bluish-green color of the spores began to appear.

PREPARATION OF ENZYM POWDER.

Experiments showed that the intracellular enzymes are present in greatest amount just before the fruiting stage is reached. By this time the sugar, which was completely inverted some days previously, is nearly used up. The reaction, which was at first acid, is now neutral or slightly alkaline to litmus. If now the culture is allowed to go on still further and the surface of the colony to become covered with spores, the enzymes are rapidly liberated into the medium and the mycelium is found to be less active.

Just as the colored spores began to appear, therefore, the mold was removed and treated according to Albert and Buchner's method for preparing "acetondauerhefe." The method as adapted to these experiments is as follows: The whole mycelium, which can be removed from the flask in the form of a thick disk, is immersed for a few moments in running water, then squeezed to remove as much as possible of the water, and run through a hashing machine. The wet mass thus obtained is immersed for ten minutes in a large volume of acetone, with constant stirring; then it is filtered off with suction. It is now immersed again for two minutes in a fresh quantity of acetone and filtered as before. The third time it is immersed in ether for three minutes, sucked as dry as possible, and spread out in a thin layer for several hours until the odor of ether is no longer perceptible. A coarse dry powder results, which is ground still finer by a suitable grinding machine.

COMPOSITION OF ENZYM POWDER.

This powder contains, besides the enzymes, all the constituents of the cells that are not soluble in acetone and ether. Needless to say, the osmotic pressure produced within the cells by this treatment

ruptures the cell walls, so that the enzymes can easily be extracted by subsequent treatment with water. The lipoids, however, have been removed almost completely by the acetone and ether. The acetone from the first extraction has a bright yellow color and when distilled until aqueous vapors begin to pass over yields a flocculent precipitate which has an odor like that of some of the terpenes. The extracted powder is white or greenish in color and is practically anhydrous. It easily becomes electrified so that the particles tend to fly apart. It contains 4.50 per cent ash and 4.35 per cent nitrogen. The greater part of the nitrogen is not in the form of true protein, but consists of chitin or nitrogenous cellulose. About 1 per cent of nitrogen can be extracted by water. The aqueous extract has an opalescent appearance, and gives the biuret, Millon, and Hopkins-Cole reactions, though rather feebly.

EXPERIMENTAL METHODS.

In all the experiments about to be described, unless otherwise indicated, this powder was used either in suspension or in the form of an aqueous extract as the source of enzyme. The advantage of this method is that a preparation of uniform activity can be used in a whole series of experiments. The powder when kept out of the light and in well-stoppered bottles seems to retain its activity indefinitely. A specimen more than a year old is apparently as active as it was when freshly prepared.

The digestions were all carried out in a thermostat maintained at a temperature of 35 to 37° C. Two to 5 per cent of toluene, depending upon the amount of surface exposed, was used as an antiseptic, unless otherwise indicated. All the tubes and flasks were tightly stoppered to prevent any change in volume by evaporation and any loss of toluene. Whenever aliquot portions were taken for analysis the mixture was first cooled down to laboratory temperature. The time of digestion varies in the different experiments, but is stated in each case. Control experiments were usually made, either with a mixture of zymolyte and boiled enzyme, or else with zymolyte and unboiled enzyme separately.

ENZYME STUDIES.

PROTEASE.

Extracellular protease, cheese ripening.

The economic importance of *Penicillium camemberti* depends upon its proteolytic enzyme. In the manufacture of Camembert cheese the mold makes its appearance on the surface of the cheese soon after its removal to the ripening room. The mold continues to develop, and about the time the fruiting stage is reached a softening of the

curd appears just under the mycelium. This softened layer rapidly increases in depth until in four or five weeks the hard curd has assumed a soft, waxy texture throughout. The change is evidently due to an enzyme secreted by the mold and liberated into the curd mass. Investigations by the writer^a have shown that this change in the physical appearance of the curd is accompanied by chemical changes which consist in the conversion of the insoluble paracasein into soluble proteoses and peptones and amino acids.

Intracellular protease.

The proteolytic enzyme is also contained in the mold powder in the intracellular form, and its presence can readily be demonstrated. As will be seen shortly, the formation of protease is independent of the presence of protein in the culture medium. It occurs when the mold is grown upon sugar and sodium nitrate, as well as when gelatin or peptone have furnished the nitrogen. The proteolytic activity of the powder obtained as described above was tested with a number of proteins. As the soluble protein of the mold itself gives the biuret reaction and undergoes autolysis with the formation of tryptophan, these reactions could not be relied upon to show positively whether the added protein had actually been digested. The change in solubility of the protein was therefore taken as the criterion.

The first tests were made with the two proteins most commonly used in demonstrating both peptic and tryptic enzymes, namely, coagulated egg white and fibrin. In these experiments, as well as in those following, an enzyme solution was prepared by extracting the mold powder for two hours with twenty times its weight of water.

Experiment 1.—Ten cubic centimeters of the filtered extract were placed in each of three small Erlenmeyer flasks. To the first was added 1 c. c. of 1 per cent HCl, to the second 1 c. c. of 1 per cent Na_2CO_3 solution, and to the third 1 c. c. water. In each of the flasks were placed two Mett tubes of coagulated egg white. At the end of a week the tubes were examined and none of them showed any signs whatever of digestion.

Experiment 2.—The above experiment was repeated, using a small flock of fresh fibrin in place of the Mett tubes. In about three days the fibrin in the flask containing alkali showed signs of disintegration, and at the end of a week it had completely disappeared. The fibrin in the neutral medium also showed some solution, though not quite so marked. The acid fibrin was least altered. This evidence of digestion of fibrin by the mold extract is, however, offset by the fact that the fibrin in the control flasks with boiled enzyme behaved in the same way, though the rate of digestion was somewhat slower, espe-

^a Dox, Arthur W. Proteolytic changes in the ripening of Camembert cheese. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 109. 1908.

cially in the neutral flask. The disappearance of the fibrin was probably caused in part at least by leucocyte enzymes present in the fibrin.

Experiment 3.—The behavior of the mold extract toward boiled fibrin was then tested. The experiment was conducted exactly as described above, except that boiled fibrin was substituted for the fresh fibrin. At the end of a week the fibrin in all of the flasks was apparently unaltered. In the presence of acid a swelling had occurred, but no signs of disintegration.

Experiment 4.—The colorimetric method recently described by Roaf^a was also tried. This method makes use of fibrin that has been stained with Congo red and the dye rendered permanent by immersing the fibrin in boiling water. A liberation of color from fibrin carefully prepared in this manner indicates proteolysis. Ten cubic centimeters of the mold extract were used, as before, in acid, alkaline, and neutral solution, and approximately equal amounts of stained fibrin. After five days only a very slight amount of color was liberated, the intensity of which was about the same as that in the corresponding control tests.

Experiment 5.—A number of other proteins were tested with respect to their digestibility by the mold enzym. The same amount of extract was used, and the tests were carried out in acid, alkaline, and neutral media. At the end of a week the acid or alkali that had been added was exactly neutralized by an equivalent amount of alkali or acid, and in the case of the globulins the precipitation was aided by heating. The protein was then filtered on a tarred filter paper, dried at 110° C., and weighed. The control experiments are given here only where they show a decided difference from the others.

Protein tests in neutral, acid, and alkaline media containing mold extract.

Protein added, 0.1 gram.	Protein recovered.		
	Neutral.	N/20 HCl.	N/20 Na ₂ CO ₃ .
Zein.....	0.0998	0.0996	0.0987
Cryst. edestin.....	.0989	.0993	.0987
Excoelsin.....	.0990	.0991	.0970
Elastin.....	.0997	.1002	.0980
Collagen.....	.0991	.0996	.1005
Ovovitellin.....	.0978	.0982	.0974
Coconut protein.....	.0991	.0982	.0990
Casein.....	.0006	.0876	.0323
Casein (control).....	.0994	.0980	.1003

Of the eight proteins tested, casein is the only one that was appreciably digested. The activity of the enzyme toward this protein is greatest in the very faint natural acidity of the extract. The digestion is less rapid in the presence of sodium carbonate and is almost inhibited by hydrochloric acid of the strength used. In the former

^a Roaf, H. E. A new colorimetric method to show the activity of either 'peptic' or 'tryptic' enzymes. *Biochemical Journal*, vol. 3, pp. 188-192. Liverpool, 1908.

case the weakening of the enzyme probably occurred before the alkali had combined with the casein, because "nutrose," the sodium salt of commerce, was found to digest readily. The readiness with which the enzyme digests casein is not surprising, in view of the fact that this protein forms the natural habitat of the organism, and is so easily digested by the living mycelium.

Experiment 6.—As the living organism has also the power of growing on gelatin and liquefying it, the enzyme might be expected to act in the same way. The effect of the enzyme solution on this albuminoid was tested by Fermi's method with liquid gelatin.^a

In each of three tubes 5 c. c. of 20 per cent gelatin containing thymol were placed. The jelly was liquefied by warming to 35° C., then 5 c. c. of enzyme solution were added, and 5 c. c. of water. In one tube the mixture was made acid by the addition of hydrochloric acid to 0.1 per cent, to another sodium carbonate was added to 0.1 per cent, and the third was made neutral. Control tubes were prepared in the same way by adding boiled enzyme. The mixtures were left in the thermostat at 35° C. for twenty-four hours. They were then removed and cooled down to 15° C. by immersion in running water. The contents of the tubes to which the unboiled enzyme had been added remained liquid, with the exception of the acid gelatin, while those containing boiled enzyme quickly solidified to a stiff jelly.

Experiment 7.—The action of the enzyme on gelatin may be demonstrated with equal readiness by Fermi's method with tubes of solid gelatin.

To each of three small test tubes 2 c. c. of 10 per cent gelatin containing 0.2 per cent thymol were added. One was made 0.1 per cent acid with hydrochloric, another 0.1 per cent alkaline with sodium carbonate, and the third was kept neutral. Two cubic centimeters of a 3 per cent mold extract were then added to each, and the same amount of acid or alkali as the gelatin contained. A few drops of toluene were added and the tubes left at room temperature. Every twenty-four hours the level of the gelatin was marked on a strip of paper pasted on the tube. The acid gelatin showed no signs of digestion; in fact, there was a slight swelling above the mark indicating the original level. In the other tubes the linear liquefaction was as follows:

Hours.	Neutral.	Alkaline.	Acid.
	<i>Mm.</i>	<i>Mm.</i>	<i>Mm.</i>
24	4	1	
48	7	2	
72	9	2	
96	10	2	

^a Fermi, C. Reagentien und Versuchsmethoden zum Studium der proteolytischen und gelatinolytischen Enzyme. Archive für Hygiene, vol. 55, pp. 140-205. München, 1906.

Experiment 8.—It has already been shown that the enzyme does not act on coagulated egg white in Mett tubes. A solution of crystallized egg albumin was tested in order to see if different results might be obtained.

Ten cubic centimeters of the enzyme solution were used and 2 c. c. of a solution of crystallized ovalbumin. After remaining four days in the thermostat the solutions were made just faintly acid and the unchanged albumin was coagulated by boiling. This precipitate was filtered and washed, and the nitrogen determined by the Kjeldahl method.

Enzym.	Coagulum (nitrogen).		
	Neutral.	0.1 per cent HCl.	0.1 per cent Na ₂ CO ₃ .
Unboiled.....	Grams. 0.0339	Grams. 0.0357	Grams. 0.0348
Boiled.....	.0344	.0359	.0358

Neither coagulated egg white nor soluble ovalbumin undergoes any appreciable digestion.

Experiment 9.—Casein, as has been seen, is readily digested by this enzyme. In order to determine to what extent the digestion goes on, the following experiment was carried out: Ten grams of casein "nach Hammarsten" were suspended in 200 c. c. water. Five grams of mold powder were extracted for two hours with 100 c. c. water, then filtered and added to the casein suspension. The bottle containing this mixture was placed in a bath maintained at a temperature of 40° C. and shaken at frequent intervals during the day. In about two days the casein had begun to disappear. In a week it had all disappeared with the exception of a scum which remained in the layer of toluene at the surface. This scum was filtered off, dried at 100° C., and weighed. Eight-tenths of a gram was obtained and the phosphorus content was found to be 0.76 per cent. It was probably unaltered casein containing adsorbed impurities from the enzyme solution. The filtrate, which was yellow in color, was left in the thermostat until the digestion had gone on for thirty-two days. At the end of that time the total nitrogen, the nitrogen in the filtrate after precipitating with tannic acid, and the nitrogen as ammonia were determined, also the total and inorganic phosphorus. The difference between the first two determinations represents caseoses and peptones; that between the second and third represents amino acids.

Test to determine digestion of casein.

[10 grams casein, 200 c. c. water, 100 c. c. 5 per cent mold extract.]

Days.	Nitrogen as—	In 10 c. c.	Nitrogen calculated as—	In 10 c. c.
		<i>Grams.</i>		<i>Grams.</i>
0	Total nitrogen.....	0.0036		
32	Total nitrogen.....	.0444	Proteoses and peptones.....	0.0105
32	Tannin filtrate.....	.0339	Amino acids.....	.0312
32	Ammonia.....	.0027	Ammonia.....	.0027
55	Total nitrogen.....	.0444	Proteoses and peptones.....	.0060
55	Tannin filtrate.....	.0384	Amino acids.....	.0357
55	Ammonia.....	.0027	Ammonia.....	.0027
32	Total phosphorus in 50 c. c., 0.0120 gram.			
32	Inorganic phosphorus in 50 c. c., 0.0124 gram.			

The digestion of casein goes beyond the peptone stage, the principal products being amino acids. The solution gave a pink biuret reaction and a strong tryptophan reaction with bromine water. The material remaining after the analytical determinations had been made was evaporated to a sirup and allowed to stand in a cool place for several days. Characteristic crystals of tyrosin and "leucin" separated out, and these were identified under the microscope.

Experiment 10.—The proteoses and peptones which are formed as intermediary products in the digestion of casein are rapidly broken down into simpler products. Although fibrin is not acted upon by this enzyme, it was thought that the proteoses and peptones derived from fibrin might be attacked more readily. The following experiment was therefore made: Three grams of "Witte peptone" were dissolved in 50 c. c. water; 2 grams of mold powder were extracted for two hours with 50 c. c. water, filtered, and added to the peptone solution. After remaining in the thermostat for fourteen days, nitrogen determinations were made as in the preceding experiment.

Test to determine digestion of peptone.

[3 grams Witte peptone, 50 c. c. water, 50 c. c. enzyme, 14 days.]

Nitrogen as—	In 10 c. c.	Nitrogen calculated as—	In 10 c. c.
	<i>Grams.</i>		<i>Grams.</i>
Witte peptone solution:		Witte peptone solution:	
Total nitrogen.....	0.0444	Proteoses and peptones.....	0.0099
Tannin filtrate.....	.0345	Amino acids.....	.0320
Ammonia.....	.0025	Ammonia.....	.0025
Control—boiled enzyme:		Control—boiled enzyme:	
Total nitrogen.....	.0444	Proteoses and peptones.....	.0336
Tannin filtrate.....	.0108	Amino acids.....	.0096
Ammonia.....	.0012	Ammonia.....	.0012

Influence of acid and alkali.—In the experiments with Witte peptone, as well as in the casein experiment, no acid or alkali was added to the digestion mixture. The extract from the mold, however, was faintly acid to litmus, though neutral to methyl orange and dimethyl-aminoazobenzene. The acidity persisted after the digestion had gone

on for some time. To determine the effect of acids and alkalies a series of tests was made. Different amounts of decinormal acids and alkalies were taken and the total volume made up to 10 c. c. with distilled water, then 0.1 gram Witte peptone was added and 0.1 gram mold powder. After twenty-four hours in the thermostat the tryptophan test was applied, using the same number of drops of bromine water in each case. With hydrochloric, lactic, and phosphoric acids the intensity of the color decreased with increasing concentration, until with N/20 acid the test was negative. Alkalies were much less inhibitory than acids. The reaction was still positive though quite faint when sodium carbonate or ammonia was present in decinormal concentration.

Summary.—From the experiments described above it will be seen that the intracellular protease of *Penicillium camemberti* readily digests casein, gelatin, and Witte peptone, but has no action on fibrin, ovalbumin, or other native proteins. In this respect it is closely analogous to the protease discovered by Cohnheim^a in the intestines of mammals, and named by him "erepsin." Vines^b has found erepsin in a large number of plants, and has been able to separate it from the peptic enzym which usually accompanies it. According to Vines, "the ereptase of plants differs from that of animals only in that its reaction range is more extensive in the direction of acidity, and is, perhaps, less extensive in the direction of alkalinity."

At the time the protease of molds was being investigated by Bourquelot and others, erepsin had not been discovered. These authors regarded the enzym as closely resembling if not identical with trypsin. There is a possibility that the organisms studied, especially *Aspergillus niger*, secretes a small amount of peptic or tryptic enzym, enough to account for the digestion obtained in some cases with fibrin; or it may be that the fibrin itself contained proteolytic enzymes from the leucocytes.

To determine whether the liquefaction of gelatin was due to a specific "glutinas," the writer prepared an extract from a dog's intestine and tested its action on gelatin by Fermi's method, with positive results. The digestion must have been due to erepsin, for the same extract had no action on fibrin.

NUCLEASE.

In Iwanoff's paper, which is the only one in the literature having to do with the enzym of molds which acts on nucleic acid, the author states that the organisms were cultivated on media containing nucleic

^a Cohnheim, O. Die Umwandlung des Eiweiss durch die Darmwand. Zeitschrift für physiologische Chemie, Band 33, p. 451-465. Strassburg, 1901.

^b Vines, S. H. The proteases of plants. Annals of Botany, vol. 23, No. 89, p. 17. London, 1909.

acid. His results show no evidence that a mold will produce nuclease when it is cultivated upon a medium consisting entirely of carbohydrate and inorganic salts. The experiments described in the preceding pages have shown that the production of protease in *P. camemberti* is independent of the presence of protein in the culture medium. Whether nuclease is produced under similar conditions was an important point that remained to be investigated.

A specimen of yeast nucleic acid was purified by dissolving and precipitating by alcohol. Five grams of the product were dissolved in just enough dilute potassium hydroxid to give a solution neutral to litmus. Two grams of mold powder were extracted for an hour with 100 c. c. water, filtered, and the solution was added to the nucleic-acid solution. The total volume was then made up to 250 c. c. and toluene added for an antiseptic. After forty-five days in the thermostat at 35 to 37° C. the solution was tested with ammoniacal silver nitrate and magnesium mixture. Both of these reagents gave a precipitate, while in the control experiment the precipitates obtained were almost negligible. One hundred cubic centimeters of the solution were taken for the purin determination. The purins were precipitated by copper sulphate and sodium bisulphite, washed with hot water, and decomposed by hydrogen sulphid. After boiling off the excess of hydrogen sulphid the filtrate was reprecipitated by copper sulphate and sodium bisulphite. The amount of precipitate was not large enough for a separation of the purins, so the nitrogen contained in it was determined by the Kjeldahl method. Total phosphorus and phosphorus as phosphoric acid were determined in 50 c. c. portions. The results are expressed as grams per 100 c. c.:

	Gram.
Total nitrogen.....	0.1788
Purin nitrogen.....	.0663
Total phosphorus.....	.0859
Phosphorus liberated.....	.0512
Control—boiled enzym:	
Total nitrogen.....	.1788
Purin nitrogen.....	.0036
Total phosphorus.....	.0859
Phosphorus liberated.....	.0041

There can be no doubt that the decomposition of the nucleic acid was due to an enzym. And it is evident that the nuclease of *P. camemberti* is formed irrespective of the presence of nucleic acid in the culture medium. The decomposition is more complete than that obtained by Iwanoff, owing to the greater length of time through which the enzym acted, and probably also to a greater activity of the enzym preparation. Iwanoff's enzym failed to liquefy gelatin, although at least one of the organisms used by him—*A. niger*—has been shown by other investigators to contain a gelatin-liquefying

enzym. These investigations, however, are not strictly comparable with those of Iwanoff, because of the different methods of culture and the different conditions of the experiments. It will be noted that the ratio between the purin nitrogen and the phosphorus liberated is different from that obtained by Iwanoff with thymonucleic acid (see p. 21).

AMIDASE.

It was seen in the experiments under protease that the mold powder had the power of liberating ammonia from casein and Witte peptone. This effect might be attributed to a deamidizing enzym. The liberation of ammonia from the protein molecule during proteolysis is probably due to a breaking down of such amids as asparagin and glutamin, which are intermediary products, into ammonia and aspartic and glutaminic acids. If this is the case, the same enzym might be expected to hydrolyze other amids in a similar manner.

In the following experiments the enzym preparation from the sucrose medium was tested with several amino compounds. A weighed quantity (0.2 gram) of the substance was dissolved or suspended in 20 c. c. water, and 0.5 gram mold powder added. Toluene was used for an antiseptic. After several days in the thermostat the contents of the flasks were transferred to cylinders and the ammonia determined by Folin's method. The following figures were obtained:

Test to determine the decomposition of amids.

Substance.	Formula.	Days.	N/5H ₂ SO ₄ .	Control.	N as NH ₃ .
			c. c.	c. c.	Gram.
Urea.....	$\begin{array}{c} \text{NH}_2 \\ \diagup \\ \text{CO} \\ \diagdown \\ \text{NH}_2 \end{array}$	4	6.3	0.5	0.0174
Asparagin.....	$\begin{array}{c} \text{NH}_2 \\ \diagup \\ \text{CO} \\ \diagdown \\ \text{CO}-\text{NH}_2 \\ \\ \text{HOOC}-\text{CH}_2-\text{CH}-\text{NH}_2 \end{array}$	4	5.0	.2	.0144
Biuret.....	$\begin{array}{c} \text{NH}_2 \\ \diagup \\ \text{CO} \\ \diagdown \\ \text{NH}-\text{CO}-\text{NH}_2 \end{array}$	5	.4	.2	.0006
Acetanilid.....	$\begin{array}{c} \text{NH}_2 \\ \diagup \\ \text{CO} \\ \diagdown \\ \text{C}_6\text{H}_5 \end{array}$	5	.8	.8	.0000
Alanin.....	$\begin{array}{c} \text{CH}_3\text{CO}-\text{NH}_2 \\ \\ \text{CH}-\text{CH}-\text{NH}_2 \\ \\ \text{HOOC} \end{array}$	5	.7	.2	.0015
Benzamid.....	$\begin{array}{c} \text{C}_6\text{H}_5\text{CO}-\text{NH}_2 \end{array}$	5	.9	.2	.0021

Urea and asparagin are both readily acted upon. Benzamid and alanin are hydrolyzed to a considerably less extent, while the hydrolysis with biuret is doubtful.

Hippuric-acid-splitting enzym.—The mold powder was found to have the power of hydrolyzing hippuric acid into benzoic acid and

glycocoll (aminoacetic acid). The experiment was conducted as follows: Two-tenths of a gram of hippuric acid was dissolved in just enough sodium hydroxid to give a neutral reaction with litmus. The volume was made up to 20 c. c. and then 0.4 gram mold powder added. At the end of a week the mixture was filtered and the calculated amount of sulphuric acid added to combine with the sodium. This solution was shaken several times with petroleum ether in a separatory funnel, and the petroleum allowed to evaporate. A crystalline residue was left which after recrystallization from water melted at 121° C. and had the crystalline appearance of benzoic acid. In the control experiment with boiled enzyme no residue was obtained on evaporating the petroleum. On repeating the experiment and extracting first with ethyl acetate according to the method of Bunge and Schmiedeberg the weight of the benzoic acid obtained was 0.1085 gram. As 0.2 gram hippuric acid yields 0.148 gram benzoic acid on complete hydrolysis, the hydrolysis in this case is 76 per cent.

The effect of cultivating the organism on media containing amino compounds would probably be an increase in the amount of amidase produced by the mycelium. This point, however, still remains to be determined. So far as the influence of nutrition on the production of enzyme has been studied in the case of the carbohydrate-splitting enzymes, as will be pointed out later, the effect has been to increase the production of the specific enzyme necessary for the hydrolysis of the given substratum. There is no reason why the same relation between substratum and enzyme formation should not hold true also for other enzymes such as amidase.

LIPASE.

The lipolytic activity of the enzyme powder prepared from cultures of *P. camemberti* that had been grown on cane sugar was very feeble. This might be expected in view of the small amount of lipase obtained by other investigators who worked with *P. glaucum* and *A. niger*. By carefully titrating the acid formed after the enzyme had been allowed to act upon the ester, a slight lipolytic activity could be demonstrated. In these experiments an extract of the mold powder, prepared as already indicated, was used. The extract alone showed no appreciable increase in acidity after remaining in the thermostat throughout the experiment. Ten cubic centimeters of the enzyme solution were diluted with an equal volume of water and 0.2 c. c. of the ester added. A few drops of toluene were added for an antiseptic, and the mixture was left in the thermostat for three days. The solution was then titrated with decinormal sodium hydroxid, using phenolphthalein as an indicator.

Ester.	N/10NaOH.	Control.
	c. c.	c. c.
Triacetin.....	1.6	1.0
Monobutyryn.....	1.8	1.0
Amyl acetate.....	1.4	1.2
Ethyl butyrate.....	2.0	1.3
Ethyl acetate.....	1.4	1.1

In every instance there is a slight increase in acidity, though in some cases it is only very slight indeed. Glycerin monobutyryn and ethyl butyrate gave the greatest increase, while with amyl acetate and ethyl acetate the increase is much smaller.

The very slight extent to which the esters were hydrolyzed may be explained in two ways—there may be a loss of enzyme during the process of preparation, or it may be that the culture medium was unsuitable for the production of lipase. It has been claimed that during the extraction of fat from seeds a considerable part of the lipase goes over into the ether extract. According to Taylor^a lipase is insoluble in ether, but is soluble in ether containing a fatty body. If such is the case, part of the lipase may be lost when the mycelium of the mold is treated with acetone and ether. Again, the lack of lipase may be due to the fact that the medium contained no ester to stimulate the formation of the enzyme. As will be seen in subsequent experiments with other enzymes, there seems to be a greater formation of a specific enzyme when its particular zymolyte is present in the medium. By analogy, the production of lipase would be increased by cultivating the mold on a medium containing a fat or an ester.

Cultures of this organism do have the power of hydrolyzing fat under proper conditions. An experiment was made in which a suspension of pure butterfat in a solution of inorganic salts was inoculated with *P. camemberti*. The mold grew very slowly at first, but after a time the growth appeared to be normal. The butterfat changed its appearance completely, and a white mass of minute crystals was formed.

Further experiments will be conducted to determine whether the fresh mycelium is more active than the "dauer" preparation and whether the presence of fat in the medium increases the amount of lipase formed.

EMULSIN.

Just as the four enzymes already discussed are formed in the absence of their respective zymolytes from the culture medium, so emulsin is formed in the absence of glucosids. The same powder that was used in the preceding experiments was tested with respect to its glucosid-

^aTaylor, E. A. On the action of lipase. *Journal of Biological Chemistry*, vol. 2, No. 1, p. 87. New York, 1906.

splitting power. The following glucosids were used: Amygdalin, arbutin, salicin, and phlorhizin. Two-tenths of a gram of the glucosid was dissolved in 20 c. c. water, and 0.1 gram mold powder was added. After three days in the thermostat the mold powder was filtered off and the solution tested to see if hydrolysis had occurred.

Amygdalin, when completely hydrolyzed, yields dextrose, benzaldehyde, and hydrocyanic acid. All three of these products were demonstrated in the digestion mixture. A strong odor of bitter almonds at once revealed the presence of benzaldehyde. The presence of free hydrocyanic acid was shown by distilling a portion of the liquid from a test tube and testing the distillate with ferrous sulphate and ferric chlorid, whereupon a precipitate of Prussian blue was obtained. The dextrose was identified by its osazone.

Arbutin hydrolyzes into dextrose and hydroquinone. In the presence of the enzyme the solution rapidly darkened in color on account of the oxidation of the hydroquinone. When tested with Fehling's solution black oxidation products were formed, as well as a heavy precipitate of cuprous oxid.

Salicin breaks up into o-hydroxybenzylalcohol and dextrose. The former was recognized in the digestion solution by the deep violet color it gave with ferric chlorid. The dextrose was identified as before, by the formation of insoluble osazone.

Phlorhizin when hydrolyzed yields dextrose and phloretin, and the latter on further hydrolysis gives phloretic acid and phloroglucinol. After the enzyme had been allowed to act on this glucosid a reduction of Fehling's solution was obtained amounting to 0.0170 gram cuprous oxid for 0.2 gram of the glucosid. Only a partial hydrolysis was obtained in this case. The digestion mixture did not give a color with ferric chlorid, showing that there was no liberation of phloroglucinol.

From the above experiments it will be seen that three typical glucosids—amygdalin, salicin, and arbutin—are readily decomposed by an intracellular enzyme produced when the mold is cultivated on a medium containing only a carbohydrate as the source of carbon. Phlorhizin is also hydrolyzed, though less readily and less completely.

ENZYMES ACTING ON CARBOHYDRATES.

The carbohydrate-splitting enzymes present in extracts or suspensions of the mold can readily be demonstrated by several methods. The first consists in observing the change in optical rotation of the carbohydrate solution to which the enzyme had been added. Another method depends upon the increase in reducing power as determined by Fehling's solution. As only two of the ordinary hexose anhydrides—lactose and maltose—reduce Fehling's solution before inversion, this test may be applied qualitatively. The third method

depends upon the formation of an osazone separating from the hot solution with a characteristic appearance under the microscope. When this method is applied to solutions containing the more complex polysaccharids, such as starch, dextrin, and inulin, the latter must first be removed from the solution by precipitation with alcohol. The first two of these methods give very accurate results and may be used for quantitative work. The osazone method, however, is merely qualitative and may even give negative results where only a small proportion of the sugar has been inverted.

In all the enzym experiments in which the hydrolysis is measured by sugar determinations a slight correction must be made for a reducing sugar formed by the autolysis of the mold. It was found that 0.1 gram of the mold powder suspended in water and kept at thermostat temperature for three days gave 0.0060 gram cuprous oxid, while the control with boiled enzym gave no reduction at all. A number of similar experiments showed that the amount of reducing sugar formed in this way was fairly constant. The sugar was found to yield an osazone separating from the hot liquid and having the crystalline appearance and melting point of phenylglucosazone. It is not unlikely that this reducing sugar has been a source of error in the work of previous investigators.

By using a small amount of powder, say 0.1 gram, the error is reduced to a minimum. It is very small in proportion to the amount of reducing sugar formed from the added carbohydrate, and it has no appreciable effect upon the optical rotation. In most of the determinations the amount 0.0060 gram is simply deducted from the weight of the cuprous oxid obtained. This slight error may be eliminated entirely, as was done in the case of one or two experiments, by subjecting the autolyzing mold extract to dialysis in a collodion bag until the sugar formed had disappeared. This procedure seemed to weaken the enzym to some extent, and this is very likely due to a destructive action of the proteolytic enzym, for according to Vandervelde^a most enzymes do not pass through cellulose membranes. When the fresh mold or a preparation dried in the air or in vacuo without acetone or ether is used the error is somewhat greater.

The source of this monosaccharid is not yet definitely known. The mold contains one or more polysaccharids, or perhaps a hemi-cellulose, which gives an opalescent appearance to the aqueous extract, and which may be precipitated in an amorphous condition by the addition of alcohol. The red color obtained with iodine suggests the presence of glycogen. It is very probable that an enzym acts on one of these carbohydrates, liberating a monosaccharid.

^a Vandervelde, A. J. J. Ueber Diffusion von Enzymen durch Cellulosemembrane. *Biochemische Zeitschrift*, vol. 1, pp. 399-408. Berlin, 1906.

On allowing the acetone to stand after it had been used for extracting the mold by the Albert-Buchner method, needle-shaped crystals soon formed on the walls of the containing vessel. In order to determine the nature of this substance, the crystals were dissolved in water and allowed to crystallize again. On evaporating, a sweet sirup was obtained, which after several days crystallized in long needles. The crystals melted at 166°C. , did not rotate the plane of polarized light, and did not reduce Fehling's solution before or after boiling with acid. These are all properties of mannite, a hexatomic alcohol which is known to occur in many of the higher fungi.

The purpose of the experiments which follow is not only to demonstrate the presence of the different carbohydrate-splitting enzymes, but also to show the influence of nutrition upon the relative amounts of the enzymes formed. With the exception of the paper by Katz (see page 27) on the regulatory formation of amylase, that of Pottevin (see page 25) on the formation of lactase in cultures upon lactose, and a few bare statements by Duclaux, very little is known about the influence of nutrition of molds upon the relative amounts of intracellular enzymes produced. This question of nutrition was studied especially in connection with the carbohydrate-splitting enzymes of *P. camemberti*.

In testing the influence of nutrition upon the relative activities of the intracellular enzymes the following procedure was adopted: A series of culture media was prepared consisting of the usual inorganic salts and 3 per cent of another carbohydrate in place of the sucrose. The media were sterilized and inoculated in the usual way with spores of *P. camemberti*. The flasks were all kept under the same conditions of temperature and light, and the organism was allowed to reach the same stage of development in each case, then the mycelium was removed and dehydrated by the usual method with acetone and ether. The preparations obtained in this way were used throughout the whole series of experiments, and the results are therefore strictly comparable. In order to determine the efficiency of the acetone-ether method as compared with simple dehydration of the mycelium in vacuo, a preparation made by the latter method was included in the series and will be designated as "dried mycelium." Preparations from mycelia grown upon the following carbohydrates were tested: Sucrose, maltose, lactose, glucose, starch paste, and inulin. In a series of test tubes there were placed 10 c. c. of a 2 per cent carbohydrate solution and 0.1 gram mold powder from the different sources indicated. Toluene was used for an antiseptic, and the mixture was kept in the thermostat for a given length of time. Then the enzym action was arrested by plunging the tubes into boiling water. After cooling, the solutions were clari-

fied by shaking with a definite volume of alumina cream and filtering. The rotation of the resulting solution was determined in a Schmidt and Haensch triple-field polariscope, using a 100 mm. tube. The readings are expressed in degrees Ventzke. Assuming that the change in rotation is proportional to the change in specific rotation when the carbohydrate is hydrolyzed by acid, the percentage of hydrolysis obtained can be calculated roughly. Although this method of calculation may not be strictly accurate, it gives approximate values which may be used for comparison.

Having briefly outlined the methods, we now turn our attention to the data obtained by their application. The enzymes will be discussed in the same order that was followed in the historical review. As some of the rarer carbohydrates, such as trehalose, melizitose, and gentianose, could not be procured, the observations are confined to the more commonly occurring carbohydrates.

AMYLASE.

Although *P. camemberti* will grow upon media containing various forms of starch as the source of carbon, the growth is not as rapid as that obtained with the simple monosaccharids and disaccharids. At the outset the germination of the spores is somewhat delayed, but later on the organism seems to adjust itself to the medium, and finally a dense mycelium is formed and normal fruiting bodies appear. Starting with the same amount of carbohydrate in the medium, soluble starch required several days longer for its complete disappearance than did sucrose. In nine days the medium no longer gave a color with iodine, but the achroodextrins persisted four or five days longer. In the case of starch paste the hydrolysis was still slower. At the end of ten days the upper layer of the medium was clear and gave no iodine reaction, but below this there was a layer of unchanged starch. Achroodextrin is much more readily utilized, and the delay in the germination of the spores is less pronounced.

As might be expected in view of the "regulatory formation" of amylase discovered by Katz in another species of *Penicillium*, *P. camemberti* when grown upon sucrose does not produce any great amount of amylase. When a 1 per cent paste of arrowroot starch was treated with 2 per cent of its weight of mold powder from the culture grown on sucrose, a blue color was still obtained with iodine after the mixture had been in the thermostat for a week. The failure to digest starch paste could not have been due to a destruction of amylase by the acetone-ether treatment, for a similar test with a fresh culture that had been dried directly gave results that were practically negative. In this case, however, the iodine reaction at the end of a week was somewhat purplish, showing that a portion of the starch must have been converted as far as the erythro-dextrin stage.

The amylolytic activity of the enzyme preparation was more pronounced when soluble starch was used in place of starch paste. Twenty cubic centimeters of a 1 per cent solution of soluble starch when digested with 0.1 gram of the powder (from sucrose) for three days gave 0.0685 gram cuprous oxid. Deducting 0.0060 for the blank, it will be seen that the amount of reducing sugar formed, calculated as dextrose, is 0.0286 gram, or nearly 13 per cent of the theoretical yield. At the end of a week no iodine reaction was obtained. The exact time required for the starch to reach the achromic point was, however, not determined in this case. The reducing sugar was calculated as dextrose for the reason that it yielded an insoluble osazone on treatment with the phenylhydrazine reagent, and this osazone was further identified by its crystalline form as phenylglucose-azone. The maltose, which is no doubt formed as an intermediate product, is evidently quickly decomposed into dextrose by another enzyme.

The activity of the amylase is extremely small compared to that of malt or saliva. The highest dilution at which the enzyme could still be demonstrated was determined in the following way: A 2 per cent solution of soluble starch was prepared and 10 c. c. placed in a series of test tubes. An extract of the mold powder was also prepared by extracting a gram of the powder for two hours with thirty times its weight of water. Different amounts of this extract were added to the starch solution and the total volume made up to 15 c. c. From time to time the progress of the digestion was determined by testing a portion of the solution with iodine. The data are tabulated below.

Effect of various amounts of mold enzyme on digestion of starch.

Mold extract.	* Color.		
	3 days.	5 days.	7 days.
5 cubic centimeters	Red.	No color.	No color.
2 cubic centimeters	Violet.	Red.	Do.
1 cubic centimeter	Blue.	Violet.	Red.
0.5 cubic centimeter	do.	Blue.	Violet.
0.2 cubic centimeter	do.	do.	Blue.
0.1 cubic centimeter	do.	do.	Do.

A distinct change in color was noticed in the tube where an extract representing 0.007 gram of the mold powder was added to 10 c. c. of the 2 per cent starch solution and allowed to digest for a week.

It has already been shown that the mold preparation acts much more readily upon soluble starch than upon starch paste. Dextrin is still more readily digested. A specimen of "C. P." dextrin was obtained, which gave no color with iodine and no reduction with Fehling's solution, and the action of the mold powder was tested upon it. In three days a suspension of 0.1 gram mold powder in 20 c. c. of a 1

per cent solution of this dextrin yielded 0.1895 gram cuprous oxid by Allihn's method. Calculated as dextrose this would represent a hydrolysis of nearly 40 per cent, or about three times as great as that obtained in the same length of time with soluble starch.

Glycogen, on the other hand, is much less readily attacked than soluble starch or even starch paste. Two-tenths of a gram of glycogen under the same conditions yielded only 0.0019 gram cuprous oxid. There was apparently no diminution in the opalescence of the solution. On repeating the experiment and testing with phenylhydrazin no separation of osazone could be obtained.

The above experiments were all conducted with the preparation of mold that had been obtained from cultures on cane sugar. To determine the influence of nutrition on the formation of amylase the method already outlined was followed. Ten cubic centimeters of a 2 per cent solution of soluble starch were placed in each of a series of tubes, 0.1 gram of the mold from the different sources was added, and the mixture was left in the thermostat for forty-eight hours. An equal volume of alcohol was then added to precipitate the unchanged starch, the precipitation being facilitated by the addition of a little sodium chlorid. After filtering, the optical rotation was determined. The solution was then evaporated to remove the alcohol, and the residue taken up in a small volume of water. The iodine test was applied and also the reduction of Fehling's solution.

Test to determine the influence of nutrition on the formation of amylase.

Source of enzym.	Rotation.	Color with Iodin.	Fehling's test.
	<i>Ventzke degrees.</i>		
Sucrose.....	4.8	Red.....	+
Sucrose(dried mycellum).....	5.0	Violet.....	+
Maltose.....	4.2	No color.....	++
Lactose.....	3.8	Red.....	+
Glucose.....	4.6	do.....	+
Starch.....	4.8	No color.....	++
Inulin.....	3.9	Violet.....	+
Blank.....	.4	Faint blue.....	-

The rotation in this case does not show the relative amount of hydrolysis. This is because the amount of dextrin not precipitated by alcohol at first increases, giving an increase in rotation, then the dextrin undergoes hydrolysis and the rotation decreases. It is therefore impossible to determine from the rotation alone whether the latter is increasing or decreasing. But the rotation and the iodine test together afford a satisfactory method of comparison. The mold obtained from the cultures on maltose, and more especially on starch, show the greatest amylolytic activity. In both of these cases the achromic point has been reached, and the reduction of Fehling's solution is stronger than that obtained with the other preparations.

In general it may be said that the amylolytic power of the mycelium of *P. camemberti* is rather feeble when the organism is cultivated upon cane sugar. Soluble starch is more readily digested than starch paste, and achroodextrin still more readily, while glycogen is only very slightly hydrolyzed. A more active preparation of amylase may be obtained by introducing starch or maltose into the culture medium in place of the cane sugar.

INULASE.

The enzyme powder prepared from cultures of *P. camemberti* on sucrose had very little action on inulin. When inulin solutions were treated with a suspension of this powder for several days, then freed from the unchanged inulin by alcohol and tested with the phenylhydrazin reagent, only a very slight separation of osazone resulted. This was regarded as evidence of digestion, however, as no separation of osazone could be obtained in the control. Several repetitions of this test gave similar results. Quantitative determinations confirmed the evidence obtained by the phenylhydrazin test. One-tenth of a gram of the mold powder was suspended in 20 c. c. of a 1 per cent solution of inulin, and the mixture allowed to digest for three days. The amount of cuprous oxid then obtained by Allihn's method, after deducting the blank, was 0.0342 gram. This represents 0.0149 gram levulose, or a hydrolysis of about 7 per cent.

The variations in the amount of inulase that might be caused by differences in nutrition were determined in much the same way as in the preceding studies with amylase. Ten cubic centimeters of a 2 per cent inulin solution were placed in a series of tubes, and 0.1 gram of enzyme powder from the various sources was added. After forty-two hours the digestion was stopped and the solutions clarified by shaking with 5 c. c. alumina cream. Polarimetric determinations were made upon the filtrate, with the following results:

The influence of nutrition on the formation of inulase.

Source of enzym.	Rotation.	Approximate hydrolysis.
	Ventzke degrees.	Per cent.
Sucrose.....	-2.6	3
Sucrose (dried mycelium).....	-2.8	8
Maltose.....	-2.6	3
Lactose.....	-2.6	3
Starch.....	-2.6	3
Glucose.....	-2.6	3
Inulin.....	-5.9	92
Blank.....	-2.5	0

Here is a striking instance of the influence of nutrition upon enzym formation. All of the preparations showed some slight activity. But the mold that had been cultivated on inulin as the

only source of carbon contained many times as much inulase as those which had been grown on other carbohydrates. The organism evidently adapts itself to the medium by increasing the amount of the particular enzym needed.

Inulin as a source of carbon for cultures of *P. camemberti* gives results similar to those obtained with starch paste, already mentioned. The germination is much delayed, and at first only a scanty growth of the organism is obtained. The mold then seems to thrive after it once gets a start, and produces normal fructification, though the mycelium is not as dense as that obtained with cane sugar or maltose. The complete inversion of the inulin requires more than two weeks, and the levulose is consumed nearly as fast as it is liberated.

In studying the rate of hydrolysis of inulin by the enzym, the preparation obtained from the culture on inulin was used, as the others possessed so little activity toward inulin. One gram of the powder was extracted in the usual way with thirty times its weight of water, and different amounts of the extract were added to 10 c. c. of a 2 per cent solution of inulin. The total volume was made up to 15 c. c. After forty-six hours in the thermostat the solutions were clarified with 5 c. c. alumina cream and the rotation was determined.

The hydrolysis of inulin with various amounts of the enzym.

Mold extract.	Rotation.	Approximate hydrolysis.
	<i>Ventzke degrees.</i>	<i>Per cent.</i>
5 cubic centimeters.....	-4.6	81
2 cubic centimeters.....	-3.6	48
1 cubic centimeter.....	-3.0	29
0.5 cubic centimeter.....	-2.6	16
0.2 cubic centimeter.....	-2.2	3
0.1 cubic centimeter.....	-2.1	
0 cubic centimeter.....	-2.1	0

The amount of hydrolysis obtained in a given length of time increases therefore with increase in concentration of the enzym. As small an amount as 0.2 c. c. of a 3 per cent extract of the mycelium which had been grown on inulin gives an appreciable hydrolysis with 0.2 gram inulin dissolved in 15 c. c. water.

RAFFINASE.

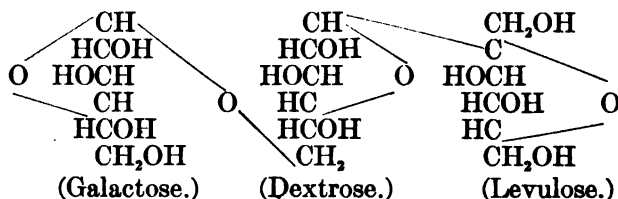
In testing for raffinase the same procedure was followed as described in the preceding experiments. Twenty-four hours' digestion of raffinose with a suspension of the mold powder yielded reducing sugars which gave a copious precipitate of insoluble osazone. Sugar determinations were made as before, after allowing 20 c. c. of a 1 per

cent raffinose solution to digest for three days with 0.1 gram mold powder. The figures are as follows: Digestion, gram Cu_2O , 0.2438; control, 0.0016; net increase, 0.2361; mold alone, 0.0061. This would represent in terms of monosaccharid a hydrolysis of about 50 per cent.

Varying amounts of a 3 per cent mold extract were added to 10 c. c. of a 2 per cent raffinose solution and the volume was made up to 15 c. c. The digestion was carried on for forty-two hours and the solutions were clarified in the usual way. The polariscope readings were as follows:

Mold extract.	Rotation.
	<i>Ventzke degrees.</i>
5 cubic centimeters.....	4.6
2 cubic centimeters.....	5.2
1 cubic centimeter.....	5.5
0.5 cubic centimeter.....	5.7
0.2 cubic centimeter.....	5.8
0.1 cubic centimeter.....	5.8
0 cubic centimeter.....	5.8

In the complex system resulting from the incomplete hydrolysis of raffinose, the percentage of hydrolysis can hardly be calculated from the rotation. The first stage in the hydrolysis is the liberation of levulose, then further hydrolysis would decompose the remaining disaccharid into dextrose and galactose, as indicated in the formula—



The mold was found to grow readily upon raffinose as the source of carbon, the appearance of the colony being much the same as that obtained with sucrose. As a sufficient quantity of raffinose was not available for the preparation of mold powder from this source, the nutritional experiments were conducted with preparations from other sources.

The influence of nutrition on the formation of raffinase.

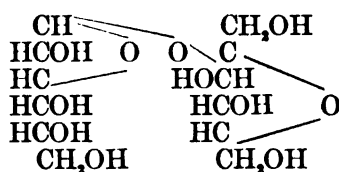
Sources of enzym.	Rotation.	Decrease.
	<i>Ventzke degrees.</i>	<i>Ventzke degrees.</i>
Sucrose.....	5.4	2.5
Sucrose (dried mycelium).....	4.8	3.1
Maltose.....	7.0	0.9
Lactose.....	3.2	4.7
Starch.....	6.7	1.2
Glucose.....	6.8	1.1
Inulin.....	5.9	2.0
Blank.....	7.9	

The decrease in rotation compared with the control shows that the raffinase was present in all of these preparations. The greatest activity was obtained with the mycelium that had grown on lactose, while that grown on sucrose was next in activity. With the other sources of sugar, the activity was considerably less. A significant fact is that both of the sugars which yielded the most raffinase contain two of the hexoses present in raffinose. The greater activity of the lactose preparation may be accounted for by the fact that the disaccharids containing galactose are relatively stable and the enzyme necessary to decompose this intermediary melibiose is increased in amount by the adaptation of the mold to lactose, which is isomeric with melibiose. The relationship between lactase and melibiase is not known, but in view of the fact that lactose and methyl-d-galactosid are apparently hydrolyzed by the same enzyme, there is some ground for supposing that this enzyme may act on other galactosids as well.

SUCRASE.

Cane sugar is an excellent source of carbon for *P. camemberti*, as well as for other molds of the same and of related genera. Owing to the ease with which it can be procured and the readiness with which most saprophytic molds grow upon it, this sugar has formed the basis of most of the culture media described. Like other molds which utilize cane sugar, *P. camemberti* first inverts it. By following the hydrolysis of the sugar by means of determinations by Allihn's method it was found that the inversion was complete in six days, after 12 per cent of the sugar had been consumed. From that time on the sugar disappeared rapidly, until after twelve days no reduction could be obtained. Not until the ninth day was there any appreciable amount of sucrase in the medium; then the amount rapidly increased.

When cane sugar is hydrolyzed a molecule each of dextrose and levulose is formed. These two monosaccharids reduce Fehling's solution, while the original sucrose does not. The hydrolysis is also accompanied by a change in rotation which will be discussed presently. The splitting takes place as indicated in the structural formula—



Qualitative tests by the usual methods showed at once that sucrase is present in the mold preparation. When a 2 per cent solution of cane sugar was treated with 1 per cent its weight of mold powder and left

in the thermostat for twenty-four hours, the resulting solution gave, on warming with the phenylhydrazin reagent, a copious separation of phenylglucosazone. It gave also a marked decrease in rotation and a strong reduction with Fehling's solution. All of these tests were negative in the control experiments.

Having demonstrated the presence of sucrase, some quantitative determinations were then made. In this series of experiments, as well as in those following, a solution of rock candy was used, as it contained no trace of reducing sugar. Five cubic centimeters of a 2 per cent solution of this sugar were measured out into each of a series of tubes. A definite volume of mold extract, prepared by macerating the mold powder with thirty times its weight of water, and filtering, was then added and the total volume made up to 10 c. c. with distilled water. After forty-eight hours in the thermostat determinations were made by Allihn's method, the copper being weighed as cuprous oxid. As 5 c. c. of this extract yielded 0.0060 gram cuprous oxid without the addition of sucrose, the error was calculated in each case and deducted before estimating the percentage of hydrolysis. The results are set forth in the following table:

The hydrolysis of sucrose with various quantities of enzym.

Mold extract.	Cuprous oxid.	Invert sugar.	Hydrolysis.
	Gram.	Gram.	Per cent.
5 cubic centimeters.....	0.2168	0.0990	94.2
2 cubic centimeters.....	.2120	.0987	93.9
1 cubic centimeter.....	.2068	.0965	92.0
0.5 cubic centimeter.....	.1833	.0858	81.7
0.3 cubic centimeter.....	.1295	.0607	57.7
0.2 cubic centimeter.....	.0825	.0387	36.9
0.1 cubic centimeter.....	.0514	.0245	23.4
0.05 cubic centimeter.....	.0354	.0173	16.5
0 cubic centimeter.....	.0000		
5 cubic centimeters enzym alone.....	.0060		
Acid hydrolysis.....	.2229	.1051	100.0

The rate of hydrolysis, except at the upper limits, follows the law of Henri.

Similar results were obtained by the use of the polariscope. While the specific rotation of sucrose is 66.5° Ventzke, the dextrose and levulose formed have specific rotations of 52.7° and -93° , respectively, and the rotation of the equimolecular mixture resulting from hydrolysis would therefore be -20.1° . As the rotation in the control experiment is 3.6° Ventzke, the calculated rotation after complete inversion would be -1.1° Ventzke. From this the percentage of hydrolysis is estimated in each case. In this experiment varying amounts of a 3 per cent extract of mold (grown on sucrose) were added to 10 c. c. of a 2 per cent sucrose solution and the total volume was made up to 15 c. c. The digestion was carried on for twenty-four

hours, then the solutions were clarified with 5 c. c. alumina cream and the optical rotation was determined as usual.

Mold extract.	Rotation.	Hydrolysis.
	<i>Ventzke degrees.</i>	<i>Per cent.</i>
5 cubic centimeters.....	-1.1	100
2 cubic centimeters.....	-1.0	77
1 cubic centimeter.....	1.5	45
0.5 cubic centimeter.....	2.4	25
0.2 cubic centimeter.....	3.2	9
0.1 cubic centimeter.....	3.4	4
0 cubic centimeter.....	3.6	0

To determine whether *P. camemberti* produces sucrase when other carbohydrates than sucrose have served as the source of carbon, mold preparations from different sources were used exactly as described under inulase and raffinase, and the rotation was measured after twenty-four hours.

Production of sucrase with various carbohydrates.

Sources of enzym.	Rotation.	Hydrolysis.
	<i>Ventzke degrees.</i>	<i>Per cent.</i>
Sucrose.....	-1.0	100
Sucrose (dried mycelium).....	-1.0	100
Maltose.....	-1.4	86
Lactose.....	-1.0	100
Glucose.....	.1	74
Starch paste.....	.2	71
Inulin.....	.2	71
Blank.....	3.2	0

The rotation after complete hydrolysis in this experiment would be -1.0° Ventzke, and from this the percentages of hydrolysis are calculated. It is evident from the table that sucrose is formed no matter which of the carbohydrates has served as the source of carbon in the culture medium. Yet sucrase is known to be a specific enzym. The highest result was obtained with the mold that had been cultivated on sucrose and lactose. The differences are by no means as great as those obtained in the experiments with inulase.

MALTASE.

The presence of maltase in the mold powder was first ascertained qualitatively by the phenylhydrazin test. A 1 per cent solution of maltose was treated for twenty-four hours with one-twentieth its weight of mold powder and an abundant precipitate of phenylglucosazone obtained by the usual test. This indicates a fairly active maltase. A quantitative determination by Allihn's method established this fact beyond doubt. In this experiment 20 c. c. of a 1 per

cent solution of maltose was treated for a week with 0.1 gram mold powder. At the end of that time the amount of cuprous oxid obtained was 0.3504 gram, while the control gave 0.2274 gram. As 0.2274 gram cuprous oxid represents 0.1779 gram anhydrous maltose and this on complete hydrolysis would yield 0.3805 gram cuprous oxid, the hydrolysis may be taken as about 90 per cent.

In determining the relative activity of different amounts of enzym, the mold that had been cultivated on maltose was used. The experiment was conducted as usual, the time of digestion in this case being twenty-nine hours. Ten cubic centimeters of a 2 per cent maltose solution were used, and diluted to 20 c. c. before polarizing. On complete hydrolysis of maltose the specific rotation decreases from 138° to 52.7° Ventzke. In the following experiment the maltose solution before inversion had a rotation of 7.1° Ventzke, and the rotation after complete hydrolysis would therefore be 2.7° Ventzke.

Mold extract.	Rotation.	Hydrolysis.
	Ventzke degrees.	Per cent.
5 cubic centimeters.....	4.6	56
2 cubic centimeters.....	5.2	43
1 cubic centimeter.....	6.0	20
0.5 cubic centimeter.....	6.6	9
0.2 cubic centimeter.....	7.0	2
0.1 cubic centimeter.....	7.1	
0 cubic centimeter.....	7.1	

As in the preceding experiments with other enzymes, the influence of nutrition on the formation of enzym was determined. The experiments were conducted as before, using a 2 per cent solution of maltose, and allowing the digestion to go on for forty-two hours. The following table shows the relation between the nutrient carbohydrate and the maltose-splitting enzym:

Effect of nutrition on the formation of maltase.

Source of enzym.	Rotation.	Hydrolysis.
	Ventzke degrees.	Per cent.
Sucrose.....	8.7	18
Sucrose (dried mycelium).....	8.4	23
Maltose.....	6.8	50
Lactose.....	8.6	20
Starch.....	6.6	53
Glucose.....	7.0	47
Inulin.....	8.9	15
Blank.....	9.8	
Calculated for complete hydrolysis.....	3.8	100

Maltase is evidently present in all of these preparations. The carbohydrates which favor its formation are starch and maltose.

LACTASE.

The action of the mold powder on lactose is very slight indeed. The phenylhydrazin test, which was first applied, showed no evidence at all of digestion. On treating a 1 per cent solution of lactose with one-twentieth its weight of mold powder, no separation of glucosazone could be obtained by the usual procedure. On the contrary, the solution on cooling yielded characteristic crystals of phenyllactosazone. Determinations by Allihn's method showed, however, a very slight increase in reducing power. Twenty cubic centimeters of a 1 per cent lactose solution were taken and 0.1 gram mold powder added. After three days in the thermostat the reducing power was determined. Two experiments in which lactose from different sources was used gave the following figures:

[Expressed in grams Cu_2O .]

	Lactose plus enzym.	Lactose alone.	Net increase.
First experiment	0.3054	0.2923	0.0071
Second experiment	.2979	.2852	.0067

Enzym alone 0.0060.

The hydrolysis is approximately 1 per cent.

The increase in rotation after inversion of lactose is so slight that a conversion of 1 per cent could not be demonstrated in this way. If a 2 per cent solution of lactose were used and the polarimetric readings made in the usual way, a change in rotation of 0.1° Ventzke would represent a hydrolysis of about 10 per cent. Obviously the error is too great for this method to be applicable. Polarimetric determinations with lactose and enzym from different sources showed no appreciable increase within the limit of error.

The organism was found to grow fairly well upon media containing lactose, and the mold powder used in some of the preceding experiments showed that the enzymes obtained from the lactose-fed mycelium were in most cases as active as those obtained from sucrose. The activity toward lactose, however, was found to be greater. An experiment was conducted in exactly the same manner as those described above, and the reducing power determined as before.

Lactose plus enzym, gram Cu_2O , 0.3251; lactose alone, 0.2883; net increase, 0.0308. Enzym alone, 0.0060.

The amount of lactase present in the mycelium grown on lactose is therefore about four times as great as that present in the mycelium cultivated on sucrose. The very small amount of lactase formed under these conditions is not surprising. This enzym is the least abundant of the three inverting enzymes in either the vegetable or the

animal kingdom. While ordinary yeast inverts sucrose and maltose, it is without action on lactose. Repeated attempts to find lactase in certain animal tissues have also resulted in failure.

SUMMARY.

By using pure cultures of a definitely identified mold and cultivating it under known conditions, the writer has endeavored to study the nature of the intracellular enzymes produced and the conditions favoring their formation. For various reasons *Penicillium camemberti* was found to be especially well adapted for such studies.

When *P. camemberti* is cultivated on a medium containing sucrose as the only source of carbon and sodium nitrate as the only source of nitrogen, a considerable number of enzymes are produced. They are retained for the most part by the mycelium until the time of fructification. By treating the mycelium with acetone and ether a dry powder results, which contains the enzymes in such form that they retain their activity indefinitely.

The powder prepared in this way was found to contain the following enzymes: Erepsin, nuclease, amidase, lipase, emulsin, amylase, inulase, raffinase, sucrase, maltase, and lactase. The lipase, inulase, and lactase were, however, quite feeble when the organism was grown on the cane-sugar medium.

The protease behaves exactly like Cohnheim's erepsin, except that its activity is greatest in neutral media or media faintly acid with acid phosphates. It digests casein, gelatin, and proteoses (Witte peptone), yielding a large percentage of amino acids, but does not attack native proteins to any appreciable extent.

The formation of erepsin is independent of the presence of protein or protein derivatives in the culture medium. An active erepsin is obtained when the organism is cultivated on a solution containing cane sugar and sodium nitrate as the only source of carbon and nitrogen.

A nuclease, liberating purins and phosphoric acid from yeast nucleic acid, is obtained independently of the presence of nucleic acid or organic nitrogen compounds in the culture fluid.

An amidase, or perhaps a group of amidases, is present, which liberates ammonia from urea, asparagin, benzamid, and alanin.

An enzym decomposing hippuric acid into benzoic acid and glycoll is present in a very active form.

The carbohydrate-splitting enzymes—amylase, inulase, raffinase, sucrase, maltase, and lactase—are formed, no matter which of the corresponding carbohydrates has served as the source of carbon in the culture medium. The amount of enzym, which in some cases is quite small, may be materially increased by cultivating the organ-

ism on the corresponding carbohydrate. This is particularly true of inulase, amylase, and lactase, which ordinarily occur only in very small amount.

There is no evidence that enzymes not normally formed by the organism in demonstrable quantities can be developed by special methods of nutrition. The influence of adding a particular substratum to the medium is, therefore, not to develop an entirely new enzym, but to stimulate the production of the corresponding enzym, which is normally formed under all conditions.

By extending the application of this principle of regulatory formation of enzym the author hopes to be able to obtain more active preparations of lipase, nuclease, amidase, and other enzymes.

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